Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 76 FEBRUARY, 1951 No. 2

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Synergistic Effect of Phosphorus^{32} and Colloidal Gold^{198} on Survival in Male Albino Rats.* (18437)

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In recent years a great deal has been published on the survival of experimental animals after exposure to ionizing radiation. These studies include not only external beta and gamma rays but radiation from internally distributed radioactive elements. Having previously studied in this laboratory the survival rate of animals to the parenteral injection of P^{32}, Sr^{89}, and colloidal Au^{198} used individually, we now wish to present results of P^{32} and colloidal Au^{198} used together. Our combination experiments were directed primarily to the study of the simultaneous injury of separate body structures or systems and to the examination of their interrelationship in producing death of the animal. It was anticipated, if any clear cut results were to be obtained from these combination studies, that it would be necessary to utilize elements having widely different tissue distributions. Although P^{32} and colloidal Au^{198} are not ideal in this respect and Sr^{89} and colloidal Au^{198} would serve this purpose even better, this choice was dictated in part by the ease in preparation and handling of the isotopes. Colloidal Au^{198}† has a half-life of 2.73 days, emits negative electrons with a maximum energy of 0.97 Mev and one gamma ray with an energy of 0.411 Mev(1). When given intravenously to experimental animals(2,3),

* Work performed under Contract W31-109-eng-78 between the U. S. Atomic Energy Commission and Western Reserve University.

† Au^{198} was obtained in colloidal form from Dr. Tabern of Abbott Laboratories, North Chicago, Ill.

1. Mitchell, A. C. G., *Rev. Modern Physics,* 1950, v22, 36.

2. Block, Walter D., Buchanan, O. H., and Freyberg, R. H., *J. Pharm. and Exp. Therap.,* 1944, v82, 391.

3. Sheppard, C. W., Wells, E. B., Hahn, P. F., and Goodell, J. P. B., *J. Lab. and Clin. Med.,* 1947, v32, 274.

and humans(3), colloidal Au^{198} shows a high concentration in the liver, less in the spleen and bone marrow, and little or none in other organs(2). Because of the insolubility of metallic gold, blood levels are low(3) and excretion is less than 1% per day following its disposition in the body(6). We have found the 15 day LD_{50} for colloidal Au^{198} in male albino rats to be 35.5 ± 3 μc/g (S.D.) of body weight(4). P^{32} has a half-life of 14.3 days and emits negative electrons with a maximum energy of 1.71 Mev. When P^{32} is administered to experimental animals intraperitoneally, in the form of disodium phosphate (Na_2HPO_4), it appears in high concentration in bone and is also widely distributed throughout other organs in the body. The concentration of P^{32}, expressed in terms of the total injected dose, rises in the bone for the first 4 days following administration and then declines gradually. In most instances the percentage of injected dose of P^{32} in other organs reaches a maximum in less than 24 hours and then declines rapidly during the first 4 days to less than 20%. P^{32} in the form of Na_2HPO_4, unlike colloidal Au^{198}, is readily soluble and actively enters into the metabolism of all cells within the body. During the first 4 days, the rate of excretion of P^{32} from the body is a rapid one, probably representing a loss from the soft tissues, then followed by a more gradual loss, the rate of which is determined by the turnover of phosphorus in bone(4). We have found the 15 day LD_{50} for P^{32} in male albino rats to be 4.5 ± 0.2 μc/g (S.D.) of body weight(4).

Experimental procedure. The studies were divided into 2 groups. In the first group the combined effects of P^{32} and colloidal Au^{198} at both ends of the LD-scale were studied. Dosages were calculated by extrapolating to LD_{20} and LD_{80} from LD_{50} studies of these agents(4). After the results were obtained for studies at both ends of the scale, a second study was then arranged using dosages which theoretically should have fallen between the 2 previously chosen dosages. 252 male albino rats, (C. F. Wistar strain) of approximately the same age and weighing between 116-177 g, were used. The animals were divided by random selection into sub-groups as indicated in the tables. They were fed a diet of Friskies and water and were housed in separate cages in air-conditioned animal quarters. The P^{32}, which was obtained from the U.S. Atomic Energy Commission, Oak Ridge, Tenn., was adjusted to a pH of 7.0 with sodium hydroxide and its activity measured immediately prior to injection. All injections were given intraperitoneally and ranged between 0.1 and 0.4 cc per animal. The activity of the colloidal Au^{198} was measured immediately prior to injection and all injections were given intravenously. Following injection the animals were returned to their cages and observed for survival for a period of 20 days.

Results. The results of Groups I and II with the dosages and survival data are en-

TABLE I. Effect of Colloidal Au^{198} and P^{32} Used Singly and in Combination on Survival in Male Albino Rats.

Dosage	No. animals	No. dead	% dead	
			Found	Expected
Au^{198} 30 μc/g	24	3	13	—
P^{32} 3 μc/g	24	6	25	—
Au^{198} 15 P^{32} 1.5 μc/g	24	16	67	19
Au^{198} 40 μc/g	24	10	42	—
P^{32} 4.6 μc/g	24	15	63	—
Au^{198} 20 P^{32} 2.3 μc/g	24	23	96	52

TABLE II. Effect of Colloidal Au^{198} and P^{32} Used Singly and in Combination on Survival in Male Albino Rats.

Dosage	No. animals	No. dead	% dead	
			Found	Expected
Au^{198} 30 μc/g	12	1	8	—
P^{32} 3.3 μc/g	12	3	25	—
Au^{198} 15 P^{32} 1.65 μc/g	12	11	92	17
Au^{198} 34 μc/g	12	8	67	—
P^{32} 3.7 μc/g	12	7	58	—
Au^{198} 17 P^{32} 1.85 μc/g	12	12	100	68
Au^{198} 38 μc/g	12	11	92	—
P^{32} 4.2 μc/g	12	8	67	—
Au^{198} 19 P^{32} 2.1 μc/g	12	12	100	78

4. Bonte, F. J., Storaasli, J. P., and Friedell, H. L., Unpublished data.

tered in Table I and II respectively.

Discussion. Colloidal Au^{198} and P^{32} when used in combination have a clear cut synergistic effect in producing death in the albino rat. Doses of each which individually produce a low death rate, result in a markedly increased mortality when administered in combination. Theoretically, if complete additivity exists, half of a definitive dose of radiation A plus half of a definitive dose of radiation B should give an effect equal to a definitive dose of A or B used singly. As may be seen in Table I, dosages of $\frac{30}{2}$ μc/g of colloidal Au^{198} and $\frac{3.0}{2}$ μc/g of P^{32} which on a purely additive basis should have resulted in death of 19% of the animals, actually produced death in 67%. Zirkle(5) tabulated the available data on various types of radiation administered in combination and noted that they were either incompletely or completely additive and that no instance of synergism had been observed. The studies presented here do not actually conflict with Zirkle's data, since the experiments with which he concerns himself are those in which the radiations are dissimilar but the tissues irradiated are the same. Here we present information in which essentially the same radiations are administered in combination, but the distribution of the radiations in tissues is different. In making reference to synergism, we refer to the biological effect on the total organism rather than the biological effect on any specific tissue.

The potentiating effect appears to be decreased at the upper end of the survival curve. This apparent decrease at the upper levels is due to the fact that for all combinations utilizing doses above the LD_{50}, the curve is sharply compressed by the limiting LD_{100}. At the present time there is nothing to indicate that the variation in energies or half-lives is related to the observed synergism.

As suggested previously, the most important difference and probably the clue to the potentiation is the difference in distribution of these two agents. Colloidal Au^{198} when given intravenously is removed from the circulation by the cells of the reticuloendothelial system. Though these cells are widely distributed, the bulk of the colloidal Au^{198} is removed by the liver (Kupffer cells), spleen, and bone marrow. P^{32}, though widely distributed at first, is retained most heavily in bone and bone marrow.

When these two agents are given in combination the reticuloendothelial system and the bone marrow with their specialized functions are simultaneously injured. The interrelation between these two systems at present is obscure but some speculation is justified in view of the findings by Jacobson(6). He has shown that protection of the spleen during radiation of the animal results in a sparing effect on the hematopoietic system and a correspondingly greater survival rate. It is attractive to postulate that injury to the

TABLE III. Energy Calculations in Ergs per g of Tissue* with LD_{50} Doses of P^{32} and Colloidal Au^{198}.

			Combination	
			Au^{198}	P^{32}†
	P^{32}	Au^{198}	2	2
Femur‡	9.58×10^5	0.717×10^5	5.149×10^5	
Spleen	1.48×10^5	5.25×10^5	3.37×10^5	
Liver	1.36×10^5	35.9×10^5	18.63×10^5	

* Calculations were made on distribution data obtained on the third day following injection of colloidal Au^{198} and P^{32}.

† It is assumed that the distribution of P^{32} and colloidal Au^{198} will not be varied when these agents are used in combination.

‡ Bone and bone marrow are included together in determining percent of injected dose per gram of tissue in bone.

Energy calculations were made using the following formula:

$D = MP\ (1\text{-}C_{15})\ (2.22 \times 10^6)\ (1.44 \times T_b)\ E\ (1.6 \times 10^6)$ where:

D = ergs per g of tissue.
M = total LD_{50} dose in μc.
P = %/g of total inj. dose in organ.
C_{15} = decay factor for 15 days.
2.22×10^6 = disintegrations/min./μc.
$1.44 \times T_b$ = biological average life in minutes.
E = average beta energy in Mev.
1.6×10^6 = ergs per Mev.

5. Zirkle, R. E., *Am. J. Roentgenol.*, 1950, v63, 170.

6. Jacobson, L. O., Simmons, E. L., Bethard, W. F., Marks, E. K., and Robson, M. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 455.

spleen and other parts of the reticuloendothelial system aggravates the injury to the hematopoietic system. A dose ordinarily producing mild injury to the blood forming system evokes, therefore, a severe depression of the blood forming tissues and an increased death rate.

Analysis of energy calculations (energy absorbed per gram of tissue) will probably not be fruitful at this time since the biological effect is dependent not only upon the radiation dosage but upon the radiosensitivity of the tissues. In addition, a great deal of information can be obtained by future experiments. However, these calculations were made and are entered in Table III. It may be seen that the liver and its reticuloendothelial system is very intensively irradiated when Au^{198} singly or Au^{198} and P^{32} combined are given. This would suggest that the liver injury is an important factor in producing the increased death rate.

With doses at the LD_{50} level, the spleen and bone marrow are severely damaged histologically by both colloidal Au^{198} and P^{32}. The liver, however, is only slightly damaged by P^{32} alone while seriously damaged by colloidal Au^{198}.

Summary. Data have been presented on the survival of the albino rat receiving radiation from internally administered P^{32} and colloidal Au^{198}. These isotopes have been administered singly and in combination. The data clearly indicate that P^{32} and colloidal Au^{198} when administered in combination act synergistically in their killing effect. This appears to be related to the simultaneous injury of the reticuloendothelial system and the blood-forming tissues.

We wish to acknowledge the technical assistance of Marvin Berg, Herbert Whanger, Martha Blodgett, and Nancy Shaw.

Received September 18, 1950. P.S.E.B.M., 1951, v76.

Further Studies on Human Hetero-Hemagglutinins for Mouse Erythrocytes.* (18438)

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Natural agglutinins for mouse erythrocytes occurring in human sera have been studied by Gorer(1), and Cohen, Winokur, Kuhns and Figge(2). Gorer(1), using Blood Group A serum, was able to demonstrate antigenic differences in mice by the use of agglutination and adsorption technics. Cohen *et al.*(2) reported that the presence or absence of the "Mo agglutinin," the term they used to designate this hemagglutinin, seemed to divide human sera into two groups independent of their major blood groups. Since only 37 individual sera were studied in the previous work(2) a more extensive investigation was undertaken to obtain more accurate knowledge of the distribution of the Mo agglutinin. This extended investigation has demonstrated that naturally occurring hemagglutinins for mouse erythrocytes are present in all human sera tested. The previous failure to demonstrate this agglutinin in all sera(2) probably resulted from the use of a technic which did not detect positive reactions in sera with low agglutinin titers.

Materials and methods. Four hundred human blood samples were obtained from the Baltimore Rh-Typing Laboratory and the Blood Bank of the University Hospital, Baltimore, Md.† The blood was allowed to clot

* This work was supported by grants from the Anna Fuller Fund and the American Cancer Society, Maryland Division.

1. Gorer, P. A., *J. Gen.*, 1936, v32, 17.

2. Cohen, I., Winokur, G., Kuhns, W. J., Figge, F. H. J., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 548.

and the serum was separated by centrifugation and inactivated by heating in a water bath at 56°C for 30 minutes. Merthiolated serum, 1:5000, was prepared by adding one part 1% solution of sodium ethyl mercuri thiosalicylate‡ (Merthiolate) to 49 parts of serum. Cell suspensions were prepared by placing 2 to 3 drops of blood (obtained from the cut tail of a mouse of the LCSa§ strain) in 2 cc of 0.9% sodium chloride (saline) solution and mixing thoroughly to produce a constant depth of color as judged by the eye. All agglutinations and titers were carried out at room temperature in 10 x 100 mm serological tubes on serum from 1 to 21 days of age. Some of the agglutination tests were made using the method used previously by Cohen *et al.*(2). This agglutination test will be referred to hereafter as the 1:6 serum dilution test, or the 1:6 test. A second agglutination method, hereafter called the 1:2 serum dilution test or the 1:2 test, was performed by placing 2 drops of serum and 2 drops of cell suspension in a tube. These were mixed by shaking and then centrifuged, following which they were read immediately. Agglutination tests were read both macroscopically and microscopically. If definite agglutination could be detected macroscopically, the microscopic examination was omitted and the test called (3+). If many large and small clumps or numerous small clumps of cells could be seen microscopically, and when such clumps could not be defined macroscopically after gentle shaking of the centrifuged specimen, it was read as (2+). If a moderate number of clumps, containing 3 or more cells were found in many fields, it was called (1+). Where evidence of agglutination was indicated in many fields by only 2 or more clumps of only 2 to 4 cells, the reaction was considered to be plus-minus (±). Negative (−) reactions showed no evidence of agglutination. In most cases (−), (±), and (1+) reactions were checked by more than one observer. Titers were determined by placing in tubes, 0.1 cc of serum in serial dilution so that the first tube contained serum undiluted; the second tube, serum diluted 1:2; the third tube, serum diluted 1:4, and so on. All dilutions were made with 0.9% saline. Addition of 0.1 cc of cell suspension made a final volume, in all cases, of 0.2 cc. The serum and cell suspensions were mixed by shaking and then centrifuged. Readings were made as soon as possible following centrifugation. Titers were expressed as the reciprocals of the fraction which indicated the highest serum dilution at which agglutination could be detected. In cases in which (±) reactions were found as the end-point of a titer, the titer was recorded as this number and also the number of the preceding tube. Thus, if a (±) reaction was found in a serum dilution of 1:256, the titer was recorded as 128-256. In agglutination and titer procedures, centrifugation was carried out in an angle (52°) centrifuge at an acceleration range of from 355 to 370 x gravity for 3 minutes. Gorer has pointed out that agglutination of mouse erythrocytes will take place in saline controls if centrifugation is too long or too fast(1). Following preliminary experiments, saline controls were run on all cell suspensions. No agglutination was produced in these controls with the above procedure.

Results. In the preliminary investigation, 53 samples of adult human serum were examined for agglutination by the 1:6 serum dilution test. In each case, 2 samples of serum were run simultaneously. Six of these sera showed enough difference between the 2 identical samples of serum to give a confusing picture, since no clear cut line of demarcation between certain of the positive and negative agglutination reactions could be demonstrated (Table I).

Sera examined by the 1:6 serum dilution test had to contain sufficient antibody to give a positive agglutination reaction in a dilution of 1:6 in order to detect the agglutinins

† We wish to express our appreciation to the Misses E. Jahn and M. Kemp of the Rh Laboratory, and Miss E. Boyle of the University Hospital, for contributing most of the sera used in this work.

‡ Obtained from Eli Lilly Co.

§ LCSa is a strain developed in our laboratory from an A x CBAN cross. The latter mice were progeny of mice obtained from Dr. L. C. Strong in 1941.

TABLE I. Agglutination Variation in 15 Sera Run Simultaneously by the 1:6 Test in Preliminary Experiments.

Serum No.	1st trial	2nd trial	Serum No.	1st trial	2nd trial
R			R		
1	1+	2+	28	2+	±
2	1+	3+	30	2+	—
3	+	—	40	—	±
4	—	—	67	1+	—
8	—	—	73	2+	1+
9	—	—	74	2+	1+
11	±	—	75	3+	1+
24	±	±			

Key to Tables I, II, and III.

— No evidence of agglutination.

± Evidence of agglutination indicated in many fields by only 2 or more clumps of only 2-4 cells.

1+ A moderate number of clumps, containing 3 or more cells found in many microscopic fields.

2+ Many large and/or small clumps seen microscopically when such clumps could not be defined macroscopically, after gentle shaking of centrifuged specimen.

3+ Macroscopic clumping seen.

present. Thus, the limits of discrimination of the 1:6 test were too near the titer end points of many of the sera. The 1:2 test was developed, therefore, so that agglutinins present only in sufficient quantities to give a titer of 2 could be detected, and results obtained with the 2 tests were compared. Three hundred and forty-seven samples of blood representing the major blood groups, both Rh positive and Rh negative, obtained from white and Negro individuals of both sexes were examined. In 38 instances, 2 samples were obtained from the same individual. A total of 392 examinations, using both the 1:6 and 1:2 test, were performed on these sera. As determined by the 1:6 test, 16 sera were negative, 5 gave (±) reactions, 8 gave (1+) reactions, 54 gave (2+) reactions, and 264 gave (3+) reactions. There were no negative results with the 1:2 test, only 1 (±) reading,‖ 4 (1+) reactions, 37 (2+) reactions and 305 (3+) reactions (Table II). Of the 26 sera on which more than one determination was made, results obtained with the 1:2 test varied sufficiently in only one case to make it doubtful whether agglutinins were present or not,‖ while 8 sera gave confusing results with the 1:6 test. In cases where more than one sample of blood was obtained from one individual, comparison of the results of these two samples yielded similar information. In the 392 examinations performed, the 1:2 test gave weaker agglutination reactions than the 1:6 serum dilution test in only 13 cases.

The Mo agglutinin titer of 29 sera was determined (Table III). Of 10 sera having titers of 8 or below, only 2 gave indication of agglutination in the 1:6 test. Of the 19 sera having titers of 8-16 or above, 4 had reactions in the 1:6 test which were not clearly positive. Only 2 of these sera had titers as high as 16. This demonstrated, further, that the 1:6 test did not detect agglutinins in sera with low titers.

TABLE II. Comparison of Agglutination Reactions Obtained with 347 Sera Using the 1:2 and 1:6 Test.

	No. of sera showing each degree of agglutination					
Test	—	±	1+	2+	3+	Total
1:6	16	5	8	54	256	347
1:2	0	1*	4	37	305	347

* This serum was subsequently checked and gave a positive reaction with the 1:2 test, although the 1:6 test had a persistent negative reaction.

TABLE III. Titers of Mo Agglutinin Content Compared with Results Obtained in the 1:6 and 1:2 Test.

Serum No.	1:6 test	1:2 test	Titer	Serum No.	1:6 test	1:2 test	Titer
B				B			
301	3+	3+	256	601	2+	2+	16
298	3+	3+	256	272	2+	3+	8-16
585	3+	3+	256	592	—	3+	8-16
583	3+	3+	256	590	—	3+	8-16
584	2+	3+	128-256	598	2+	2+	8
305	3+	3+	128	594	—	3+	8
600	2+	2+	64-128	593	—	2+	8
307	2+	2+	32- 64	288	2+	3+	4-8
278	3+	3+	32- 64	591	—	3+	4
587	2+	3+	32	268*	—	±	4
597	2+	2+	32	602	—	3+	2-4
296	2+	3+	16- 32	596	—	3+	2-4
261	3+	3+	16	279	—	1+	2
297	±	3+	16	595	—	3+	<2
300	±	3+	16				

* This serum was subsequently checked and gave a positive reaction with the 1:2 test, although the 1:6 test had a persistent negative reaction.

‖ This serum was subsequently checked and gave a positive reaction with the 1:2 test although the 1:6 test had a persistent negative reaction.

In the previous work(2), sera which were tested for Mo agglutinins were preserved with Merthiolate. To determine whether Merthiolate had a destructive effect on this agglutinin, 16 sera were collected and within 24 hours were inactivated and divided into 2 portions, to one of which Merthiolate was added as described previously. These sera were then stored in a refrigerator for periods ranging from 6 to 19 days following which the merthiolated and unmerthiolated sera were tested simultaneously. The difference between titers of serum with and without Merthiolate never varied more than one tube. In 5 cases, the titer of merthiolated serum was ½ tube lower than the serum without Merthiolate. In one case, the titer of merthiolated serum was one tube higher and in another case ½ tube higher than the serum without Merthiolate.

Discussion of results. The Mo agglutinin, a naturally occurring human hetero-hemagglutinin for mouse erythrocytes, was found in all the 400 sera tested in this investigation. The previously reported(2) failure to detect agglutinins in sera of some human subjects was undoubtedly due to the fact that the method did not detect agglutinins in sera with low titers. Waller(3) has shown that Merthiolate, in small concentrations, inhibits the action of anti-Rh agglutinins. If this were true of the Mo agglutinin, it would constitute additional reason for the large number of sera which were reported as not containing the agglutinin in the previous investigation. Results obtained in the merthiolated series of sera seemed to indicate that there might be a small degree of inactivation of the Mo agglutinin by Merthiolate. However, this inactivation was not of sufficient degree to account for the results obtained previously. Age, sex, color or major blood groups were not related to the degree of agglutination exhibited by the sera tested. Gorer(1) has used what, in all probability, is this agglutinin in detecting antigenic similarities and differences in various strains of mice. Thus this agglutinin was used to detect what he has called Antigen III. The question of whether the Mo agglutinin may have some significance comparable to the heterophilic antibody found in infectious mononucleosis should be investigated.

Summary. The Mo agglutinin (a human agglutinin for mouse erythrocytes) was found to be present in 400 human sera tested. The failure of previous attempts to demonstrate the agglutinin in all sera was probably due to the fact that the method used was not sufficiently sensitive. Merthiolate has little or no inactivating effect on the Mo agglutinin. The significance of the variation in amount of Mo antibody in human sera has not been determined.

3. Waller, R. K., *Am. J. Clin. Path.* (tech. section), 1944, v8, 116.

Received October 10, 1950. P.S.E.B.M., 1951, v76.

Neurotropic Viruses in Extraneural Tissues. III. MM Virus in Human and Murine Testicular Tissues.* (18439)

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In previous papers of this series(1,2), MM virus has been shown to multiply in the following tissues of hamsters and/or mice: soft tissue of the hind foot, skeletal muscle, skin, pharyngeal wall, urinary bladder wall, and embryonic tissues. All of the specimens of tissue in which virus was shown to multiply contained either smooth or striated muscle.

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Evans, C. A., and Chambers, V. C., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 436.

2. Chambers, V. C., Smith, W. M., and Evans, C. A., *J. Immunol.*, 1950, v65, No. 6.

From these studies and the investigations of others it appears that MM and the closely related viruses, Columbia SK and EMC, are characterized by pronounced myotropism as well as by their well known neurotropism.

In attempts to determine whether MM virus will multiply in extraneural cells other than muscle, we have used principally lymph nodes and testes. Up to the present, inconclusive results have been obtained with lymph nodes. In this paper it will be shown that MM virus multiplied in human and murine testicular tissue *in vitro*. Attempts to demonstrate multiplication in murine testes inoculated *in situ* gave less conclusive results.

Experimental methods. The virus employed was from the same source, and the experimental animals were from the same colony as described in previous studies(1,2). Tissue culture methods were the same as those described in previous papers(2,3). Approximately 0.04 ml of minced tissue was incubated in 4 ml of a fluid medium composed of bovine serum ultrafiltrate diluted with 3 volumes of Hank's solution with 100 units of penicillin G and 100 μg of streptomycin or dihydrostreptomycin per ml. The flasks were flushed with 5% CO_2 in air, closed with rubber stoppers, and incubated at 36° to 37°C. After each 3 or 4 days' incubation, 3.6 ml of medium were removed from each flask and replaced by an equal volume of fresh medium to provide a continuous supply of nutriments. All cultures were prepared in triplicate and tests for virus were made using pooled fluid from all 3 flasks. Virus content of specimens was determined by preparing serial 10-fold dilutions in Ringer's solution containing 0.5% glucose and injecting 3 mice approximately 1 month old intracranially with 0.03 ml of each dilution. All mice that survived the first 24 hours and died within 7 days were considered to have died of infection with MM virus. Checks for bacterial contamination of the inoculum were made routinely on Proteose peptone No. 3 agar, tryptose agar or veal infusion agar. During intervals of storage specimens were kept in sealed glass tubes in a dry ice chest.

Results of experiments on multiplication of MM virus in testicular tissues of mice. In 2 experiments a total of 13 testes of mice approximately 12 weeks old were inoculated with between 100 and 1000 minimal infective doses (m.i.d.) of MM virus and subsequently removed at 2, 7, 24, or 48 hours and assayed for virus. Virus was present at 24 hours in titers of 10^{-2} to 10^{-3}; at 48 hours, 1 testis gave similar results but 2 of the 3 testes removed at this time contained no detectable virus. These results suggested that the virus might have multiplied locally in some but not all testes. Whether the multiplication occurred in muscle cells of the scrotum, the cremaster, sustentaculum, or in nonmuscular tissues could not be determined. In experiments on propagation of MM virus *in vitro* in mouse testicular tissue, white mice approximately 7 months old were used. Virus with an LD_{50} of $10^{-5.25}$ was added to the tissue in each flask in an amount sufficient to give a 10^{-5} dilution. Tests at 3, 6, 10 and 13 days showed that the largest amount of virus was present at 3 days; no virus was detected at 10 and 13 days.

An experiment was carried out in which 9 serial subcultures of virus were made with incubation periods of 3 or 4 days in each tissue and occasional periods of storage of fluid medium in the frozen state. Tests for virus in medium at the end of each 3- or 4-day interval were always positive. The calculated dilution of the original inoculum in the 9th serial passage was 10^{-13}. Fluid medium from this culture killed all 3 mice injected with the 10^{-2} dilution and 1 of 3 mice injected with the 10^{-3} dilution. It is therefore evident that MM virus multiplied extensively in this series.

In checking the identity of the virus present in the last tissue culture of the series, it was shown to be resistant to ether treatment overnight in the refrigerator and to cause paralytic infection after intramuscular inoculation of mice and hamsters. Bacteriological tests were negative.

Results of experiments on multiplication of

3. Smith, W. M., Chambers, V. C., and Evans, C. A., *Northwest Med.*, 1950, v49, 368.

TABLE I. Tests for MM Virus in Tissue Cultures of Human Testes.

Tissue culture*	Culture age, days	Tissue age, days	Inoculum age, days	Calculated dilution of inoculum	Fate of mice inj. with dil. of tissue culture fluid				
					10^{-0}	10^{-1}	10^{-2}	10^{-3}	
T_1F_1	1	1	1	10^{-5}	3 4 4‡	s s s	s s s	s s s	
	2	2	2	10^{-5}	s s s	s s s	s s s	s s s	
	3	3	3	10^{-5}	(4) 8 s	s s s	s s s	s s s	
T_1F_2	2	5	5	10^{-6}	3 3 4	3 3 3	3 s s		
	3	6	6	10^{-6}	3 4 4	3 3 4	5 s s		
†	4	7	7	10^{-6}	2 3 3	(1) 3 3	3 9 s		
T_1F_3	3	10	10	10^{-7}	3 3 4	3 4 s	s s s		
T_2F_1	1	9	8	10^{-7}	4 4 s	(12) s s	s s s	s s s	
	3	11	10	10^{-7}	3 4 5	4 7 s	s s s		
†T_2F_2	4	15	14	10^{-8}	3 3 5	3 4 4	3 s s		
T_2F_3	4	19	18	10^{-9}	(1) s s	s s s	s s s		
T_3F_1	4	4	18	10^{-9}	s s s	5 s s	(14) s s		
†T_3F_2	4	8	22	10^{-10}	3 3 3	3 3 3	3K s s		
T_4F_1	4	4	26	10^{-11}	3 3 3	3 5 s	4 s s	s s s	July 29
T_4F_2	4	8	30	10^{-12}	2 2K 3	2 3 3	3 4 4	s s s	
T_4F_3	4	12	34	10^{-13}	3 4 5	3 5 6	s s s	s s s	

* T_1 = first tissue exposed to virus. T_2 = second set of tissues exposed to virus, etc. F_1 = first fluid in a given culture. F_2 = fluid in a tissue culture after 1 change at 3 or 4 days until second change of fluid.

† Shows which fluid was used to infect each new tissue.

Culture age = time this combination of tissue, medium and virus was incubated before specimen was removed.

Tissue age = total time tissue has been in tissue culture.

Inoculum age = cumulative time virus has been in active tissue culture.

Calculated dilution of inoculum = dilution of the original inoculum that has occurred in the tissue culture at this stage.

‡ Number = day of death of mouse. S = mouse survived. No. in parentheses = death of mouse on day indicated is believed the result of causes other than MM infection.

K = mouse killed on day indicated with symptoms of MM infection.

Since tissue-to-fluid ratio was 1:100, virus content of fluid diluted 10^{-1} equals 10^{-3} in terms of amount of virus per unit mass of tissue.

MM virus in human testicular tissue. Tissue for these experiments was obtained from orchidectomy specimens from patients 57 to 78 years old, all of whom had carcinoma of the prostate gland except 1 who had benign prostatic hypertrophy. Two of them had received estrogen therapy, 1 for a period of 6 months and the other for 5 years prior to operation. Virus in an amount sufficient to give a concentration of 10^{-5} was added to the first set of cultures. Tests at 1, 2, and 3 days showed little virus. At 3 days the fluid was changed. Two days later virus was present in nearly maximum concentrations. This high level of virus was maintained for 3 days. At this time fluid was again changed. Subsequent tests showed that on the 10th day considerable amounts of virus were still present. For the 2nd tissue of this series (T_2), a tissue culture that had been maintained 8 days without virus was employed. The virus increased in amount and reached its maximum titer 7 days later, which was the 15th day of culture for this tissue. Subsequently the virus disappeared.

In the 3rd tissue and the 4th tissue, maximum amounts of virus were present at 8 days (See Table I, T_3 F_2 and T_4 F_2). The calculated dilution of the original inoculum in the 3rd fluid of the 4th tissue was 10^{-13}. The 10^{-1} dilution of this fluid contained sufficient virus to kill all 3 mice injected. Therefore it was concluded that MM virus multiplied extensively in human testicular tissue *in vitro*.

To check the identity of the virus, material from the last passage was shown to be infective after ether treatment overnight at refrigerator temperature and to cause flaccid paralysis of extremities of hamsters injected intramuscularly. One guinea pig injected intraperitoneally with 0.25 ml of the etherized fluid remained unaffected. Bacteriological tests of the fluid were negative.

Discussion. It is evident that the virus

multiplied extensively in tissue cultures of mouse testes and human testes. It is not clear whether the virus multiplied in the minute amounts of muscle from vessel walls, etc., or in some other tissue. Sections indicate that some cultures contained a small bit of striated muscle. In view of the relatively high titers of MM virus found in infected muscle-containing tissues in previously reported experiments(2) it seems quite possible that very small amounts of this type of tissue might give rise to easily detectable amounts of virus in tissue cultures.

The differences in rate of growth and period of survival of MM virus in cultures of human and mouse tissues may have clinical significance. If the slow rate of growth in human tissues *in vitro* is representative of the rate of growth *in vivo,* there should be more time for protective antibodies to be formed in infected persons than in mice or hamsters. The rapid decline in amount of virus in tissue cultures of mouse tissues may result from rapid killing of all susceptible cells, a factor that might contribute to the known severity of an infection in animals of that species.

Conclusions. MM virus multiplied extensively in 9 serial passages in mouse testicular tissue *in vitro* and in 4 serial passages in human testicular tissue *in vitro.* Fluid from the last tissue of each series represented a 10^{-13} dilution of original inoculum. These experiments do not exclude the possibility that the virus may have multiplied in the very minute amounts of muscle tissue present in the cultures. The virus multiplied more slowly and persisted longer in human tissue than in mouse tissue.

Received October 30, 1950. P.S.E.B.M., 1951, v76.

Comparison of Excretion of Bromsulphthalein and Sodium Cholate after Intravenous Injection, Separately and Combined. (18440)

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(Introduced by W. J. Darby)

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Recently, interest has arisen in comparative studies of liver function tests, and the concept of association rather than dissociation of these tests has been brought to light(1,2). Although the bile salt tolerance test has been investigated intensively(3-9), it has not been subjected to a comparison with the bromsulphthalein test. The administration of bile salts has been observed to cause a retention of bromsulphthalein(10-12), but the blood

* National Research Council Fellow, 1949-50. Preliminary studies supported by the Henry Strong Denison Scholarship in Physiological Chemistry, Johns Hopkins University School of Medicine.

† The authors are grateful for the advice and assistance rendered by Doctors John C. Burch, J. Logan Irvin, John Shapiro, and John Imboden.

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concentrations of the injected bile salts have not been studied, and the effect of the bromsulphthalein on the excretion of bile salts has not been reported. Macdonald(13) has indicated that the bromsulphthalein excretion test afforded more accuracy in determining hepatic reserve if several samples of blood were drawn at intervals rather than the usual single sample.

In order to facilitate multiple determinations of bile salts in serum, a method was sought which was not only accurate but relatively short. A modification of the procedure reported by Minibeck(14), which has been virtually unnoticed in this country, was employed. We have applied this technic and are reporting comparative data on the bromsulphthalein and the bile salt tolerance tests when conducted separately and simultaneously on dogs in health and following hepatic damage.

Methods. Full grown mongrel dogs weighing from 8 to 12 kilos were used. The sodium cholate solution for intravenous injection contained 50 mg of cholic acid per ml and sufficient sodium hydroxide to adjust the pH to 7.4. This solution contained 25% glucose for the purpose of diminishing pain and thrombosis(5). It was passed through a Selas porcelain filter, porosity 02 and stored in rubber capped vials in the refrigerator. A dose of 50 mg of cholic acid per kilo of body weight was used. The bromsulphthalein solution contained 50 mg of the dye per ml of solution.‡ A dose of 5 mg bromsulphthalein per kilo of body weight was given. For the combined cholate and bromsulphthalein injections the 2 solutions were mixed before injecting. Injections were made into a superficial leg vein and samples of blood were drawn from a different vein, usually the femoral. Cholic acid was analyzed by the following modification of the method of Minibeck(14): 8.5 ml of 95% ethyl alcohol were pipetted into a test tube etched at the 10.0 ml volume mark, and 0.5 ml of barium oxide-acetate solution was added.§ 1.0 ml of the serum to be analyzed was added dropwise with shaking. The tube was heated over a small flame to boiling, and boiling was continued for 2 minutes. The mixture was cooled and alcohol was added to the 10.0 mark. It was then filtered, and 3.0 ml of the filtrate were transferred to a centrifuge tube. The solution was evaporated to dryness under reduced pressure at 40-45°C. Approximately 4 ml of the purified ethyl acetate‖ were added to the dry residue. 0.1 ml of a mixture of calcium oxide in ethyl acetate was added.¶ The tube was heated at 90°C for 2 minutes, centrifuged, and the ethyl acetate decanted. A second and third portion of 4 ml of ethyl acetate (but no additional calcium oxide) were added and, as before, heated, centrifuged, and decanted. 10.0 ml of acetic acid reagent** were added to the residue and the solution was stirred thoroughly with a glass rod and placed in a water bath at 37-40°C for 30 minutes along with 0.2 ml of 10 mg % standard cholic acid solution†† in 95% ethyl alcohol. The Coleman No. 12 photofluorometer with primary filter B-2 and secondary filter PC-2 was employed. The standard cholic acid solution was used to set the instrument. The reading was compared to a standard curve prepared by determining the fluorescence of graded amounts of cholic acid in 10.0 ml of the acetic-sulfuric reagent. The result was expressed in terms of cholic acid per 100 ml of serum.

Recoveries of cholic acid and glycocholic acid added to human serum are given in Table

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‡ Hynson, Westcott and Dunning preparation.

§ 20 g of barium oxide, anhydrous, Merck, were added to 400 cc of water, warmed and filtered. Sufficient barium acetate was added so that the solution contained 0.4 g of barium acetate per 100 cc.

‖ Ethyl acetate was purified according to Fieser, Exp. in Organic Chem., 1941, p. 364.

¶ 3.3 g of finely pulverized anhydrous calcium oxide were mixed with 100 ml of purified ethyl acetate. This mixture was shaken immediately before using.

** Nine parts of concentrated sulfuric acid to one part glacial acetic acid.

†† Cholic acid was generously supplied by the Ames Co.

TABLE I. Recoveries of Bile Acids Added to Serum.

Bile acid	No. of determinations	Theoretical, mg %	Determinations, mg %	Avg analytical recovery, %	Avg deviation of single determination $\alpha+, \pm$ %
Cholic acid added to human serum	5	0.8	0.72	90.0	5.4
	12	4.0	3.82	95.4	4.97
	9	8.0	7.64	95.5	4.71
Glycocholic acid added to human serum	6	0.8	0.63	79.1	11.10
	6	4.0	3.75	93.8	3.4
	6	8.0	7.63	95.3	7.3
Cholic acid added to dog serum	7	0.8	0.59	73.5	13.6
	4	2.0	1.90	95.0	7.5
	8	4.0	3.59	89.6	3.7
	5	8.0	7.43	92.8	2.7
	3	37.5	40.3	107.4	2.5

α— Average deviation of single determination is calculated from the formula $\alpha = v/n$ in which v is the deviation of each determination from the mean and n is the number of determinations.

I. The desired volume of the standard cholic acid solution was evaporated to dryness in a test tube, and a known amount of serum was added, mixed thoroughly, and allowed to stand for 20 minutes. 1.0 ml of serum was then withdrawn and analyzed as described above. Recoveries of cholic acid added to dog serum are also given in Table I.

In Minibeck's procedure, the alcoholic filtrate was evaporated to dryness in atmospheric pressure. Recoveries were improved by taking the filtrate to dryness under reduced pressure at 40-45°C. In order to study the effect of the method of drying of the alcoholic filtrate on the determination, glycocholic acid was added to serum at various concentrations and the filtrate evaporated (1) under reduced pressure and (2) at atmospheric pressure on a boiling water bath. At the levels of 0.8, 4.0 and 8.0 mg %, recoveries under reduced pressure averaged 79.2, 94.0, and 95.8% of the theoretical amount respectively and, under atmospheric pressure, 50.2, 78.8, and 87.0% respectively. In another series, cholic acid was added at the level of 2.0 mg %; recoveries under reduced pressure averaged 101.2% and under atmospheric pressure 75.0% of the theoretical amount. Pure alcoholic solutions of cholic acid and glycocholic acid were not so easily affected by heat, and could be evaporated to dryness in boiling water without loss of fluorescence. The BSP analyses were made according to Gaebler(15) except that a Klett-Summerson photoelectric colorimeter was used. For the production of liver damage, carbon tetrachloride, Merck, in a dosage of 1.0 ml per kg of body weight was given by stomach tube. Approximately 13 hours after the poisoning, the BSP excretion test was done; at 16 hours the cholate excretion test, and at 20-24 hours the combined BSP and cholate tests were made. Immediately after the combined test, a needle biopsy specimen of the liver was taken. Later the livers were examined grossly and microscopically.

Results. When the values obtained on each of the first three dogs were plotted, the curves followed a similar pattern. The averaged values of each experiment on the three dogs were used, therefore, in constructing Fig. 1 and 2, in which the BSP retention in one graph and cholic acid concentration in the other were plotted against time after injection.

In the healthy animals, the cholate and the BSP excretion rate compared closely with the findings of others(3,10,13,15). When the cholate and BSP were administered in combination, the excretion of BSP was greatly retarded during the first 15 minutes. At 30 minutes, however, there was only a slight difference from the BSP retention when BSP was injected alone. The cholic acid excretion, on the other hand, appeared not to be altered by the presence of BSP in the blood.

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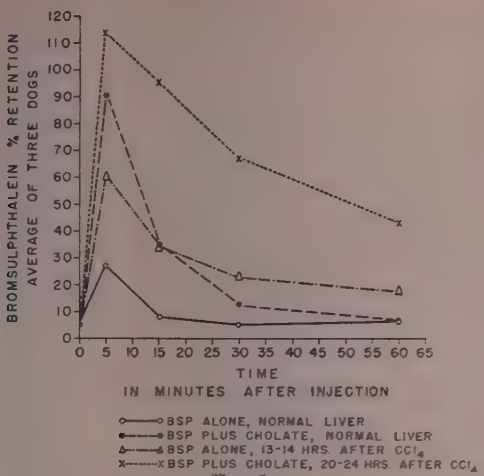

FIG. 1.

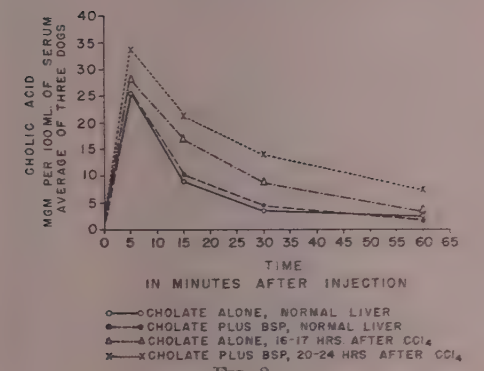

FIG. 2.

The reason for the delay in BSP excretion when BSP and cholate were given together was investigated. Since glucose was used in making the cholate solution, BSP was combined with a 25% solution of glucose adjusted to pH 7 with sodium hydroxide, and injected. The rate of the excretion of BSP was unchanged. Cholic acid, equivalent to 20 mg %, added to an aliquot of serum containing BSP, did not alter the intensity of absorption. In order to determine if mixing the BSP and sodium cholate before injection had an effect on the BSP retention, an experiment was performed on a fourth dog in which BSP was injected into a superficial vein of the right hind leg at the same time that sodium cholate was injected into a vein of the left hind leg. The retention of BSP and the concentration of cholic acid were comparable to those observed in studies in which the two solutions were mixed before injection. In this dog, as in the other 3, the BSP at 5 and 15 minutes after injection of both BSP and cholate was greatly elevated, while the cholic acid concentration was not affected by the BSP in the blood. The order of performing experiments in the first 3 dogs was: sodium cholate, BSP, and combined BSP and cholate. To determine if the sequence of injection might influence the BSP retention, the order was reversed in a fifth dog so that the first experiment performed on this dog was the combined injection of BSP and sodium cholate. The same results were observed as in the other dogs.

After the livers were damaged with carbon tetrachloride, the rate of excretion of BSP became much more abnormal than the cholate excretion rate. The BSP test in each dog definitely indicated liver disease. In the presence of liver damage, the cholic acid concentration in the serum 15 minutes after the injection was greater than one-half the 5 minute concentration. When the liver was normal, however, the 15 minute concentration was less than one-half that at 5 minutes. The cholate excretion test was performed approximately three hours after the BSP test, at which time the liver damage would be predicted to be more severe. In the combined injection tests the cholic acid concentrations in 2 dogs were somewhat higher than when cholate alone was injected. In a third dog, however, there were no significant differences. In the presence of hepatic damage, the combined test resulted in a BSP retention during the first 30 minutes which paralleled roughly that in the BSP alone but which was 35-60% higher. In 2 dogs the BSP retention in the combined test was definitely higher after carbon tetrachloride than when the liver was normal, whereas in a third animal the five minute levels were the same, but the dye was excreted slower after carbon tetrachloride had been given.

Microscopic examination of the liver biopsies taken 20-25 hours after administration of the carbon tetrachloride revealed in each

dog a coagulation necrosis with nuclear fragments and infiltration of a few leucocytes involving principally the central zone of the lobules.

Discussion. A modification of the method described by Minibeck was found to be satisfactory for the determination of cholic acid and glycocholic acid. One disadvantage of this procedure is that bile acids containing less than three hydroxyl groups did not give significant fluorescence. Desoxycholic acid had only 14% of the fluorescence of an equivalent weight of cholic acid. Chendesoxycholic acid had 30%, alpha hyodesoxycholic acid 5%, and dehydrocholic acid 2.5% of the fluorescence of cholic acid. For the purposes of determining cholic acid, however, the method was adequate. When sodium cholate was given simultaneously the excretion of BSP was greatly delayed, but there was little effect on the cholate excretion. Apparently the liver preferentially excreted the cholate before excreting the BSP. Sodium cholate and BSP may, therefore, be handled in a similar manner by the liver. It appears possible that the BSP test might be rendered even more sensitive by simultaneously giving cholic acid. Plans have been made for investigating this problem.

Lichtman(16) states that it is the consensus that the BSP is a valuable test of liver damage except when there is biliary obstruction. Mateer and co-workers(17) pointed out that in obstructive jaundice, due to back pressure and interference with the flow of bile from the ducts, there must be a simultaneous backing up of the BSP in the serum, which is due to extra-hepatic obstruction rather than to impairment of cellular function. It has been proven that cholate concentrations reached levels as high as 13 mg % in jaundice(18). In addition to a purely mechanical interference with the excretion of BSP, it appears from the present study that the high serum concentrations of cholate in obstructive jaundice may also impede the excretion of BSP. The effect of bilirubinemia on retention of BSP is unsettled. Dragstedt and Mills(19) reported that intravenous injection of bilirubin caused a retention of BSP. Cantarow and co-workers(20), however, did not confirm this observation; on the contrary, they suggested that the BSP was excreted preferentially over the bilirubin.

Summary. 1. A modification of Minibeck's method was found to be satisfactory for the determination of cholic acid and glycocholic acid added to serum. 2. In healthy dogs when bromsulphthalein and sodium cholate were injected together, the concentration of bromsulphthalein in the serum was greatly elevated five and fifteen minutes after administration. The cholate concentration, however, was normal. 3. In early central necrosis of the liver produced by carbon tetrachloride, the bromsulphthalein excretion was definitely delayed while the cholate excretion was only slightly changed. The bromsulphthalein test appeared to be more sensitive in detecting early hepatic damage. During the first thirty minutes the bromsulphthalein retention in the combined test paralleled roughly the retention when bromsulphthalein was given alone, but was 35-60% higher.

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Received November 6, 1950. P.S.E.B.M., 1951, v76.

Iron Content of Intestinal Lymph of Rats. (18441)

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Studies on the iron content of thoracic duct lymph, Moore *et al.*(1) and Endicott *et al.* (2), were acute experiments, lasting less than 24 hours. This work has been repeated using the technic of Bollman *et al.*(3), which allowed drainage of lymph exclusively from the intestinal bed and which permitted continued observation over a period of days.

Methods. Lymph was obtained by continuous drainage through cannulae of polyethylene tubing secured in the lacteal draining the intestine of Sprague-Dawley strain rats weighing about 200 g. Following intubation of the lymphatic under ether anesthesia, the animals were maintained in plastic cages(4) and allowed free access to either 0.4 or 0.9% saline as drinking water and commercial rations. No attempt was made to regulate the iron content of the diet in this group of animals. Specimens, collected over 12-hour intervals in iron-free tubes containing 1.5 ml of 2% potassium oxalate solution were measured and analyzed within 24 hours, by the method of Kitzes *et al.*(5). Strict precautions were taken to avoid contamination of reagents or glassware by iron. Dialyses were carried out in cellophane bags rendered iron-free by soaking in 0.1 normal hydrochloric acid for 24 hours followed by thorough washing with iron-free water. Specimens were dialyzed against 8-10 volumes of iron-free 0.9% saline containing acetate buffer at pH 7 and 4.5 or hydrochloric acid at pH 1.5 for 24 hours.

Results. 1. Lymph volume was found to be about one ml per hour in animals receiving 0.4% saline. (Table I.) When 0.9% saline was given, however, lymph volume increased 3- to 4-fold. Iron content of 12-hour specimens of intestinal lymph from 7 rats varied slightly from animal to animal, but was reasonably constant in the same animal for 7 days. When lymph volume was increased by giving 0.9% saline, the concentration of iron varied inversely to the volume. The corresponding output of iron in gammas per hour was slightly, but significantly, increased.

TABLE I. Iron Output from Intestinal Lymph Fistulae of 7 Rats for Periods Up to 7 Days.

Drinking water, % saline	Lymph vol. (ml/hr)	Iron output (γ/hr)	Conc. (γ/100 ml)
.4%	.94 ± 0.48*	.47 ± .28	53.74 ± 18.62
.9	2.84 ± 1.74	.88 ± .52	35.43 ± 15.68

*—Mean ± stand. dev.

TABLE II. Dialysis of Lymph.

Aliquot	pH	Lymph iron content	
		Control	After dialysis
1	7.0	35.8	34.8
2	4.7	32.6	32.6
3	1.5	28.2	9.6

TABLE III. Dialysis of Lymph Plus Added Iron.

Aliquot	Added iron γ/100 ml	Lymph iron content	
		Control	After dialysis
1	150	35.8	48.6
2	150	38.3	47.2
3	200	38.3	47.2
4	250	38.3	47.2

2. Aliquots of pooled lymph were dialyzed against buffered saline at pH 7.0, 4.7, and 1.5. As shown in Table II, the iron was not removed from the lymph samples at pH 7.0 and 4.7 but freely dialyzed at pH 1.5. From Table III it is apparent that added iron, up to 48 γ per hundred ml of lymph, is not dialyzed under these conditions at pH 7.0.

Discussion. There are 3 possible sources of

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the iron in intestinal lymph: (a) absorbed iron, (b) iron stores in intestine including lymph nodes and intestinal mucosa, (c) plasma iron. Following the administration of iron orally to dogs, Moore(1) found a greater increase of iron in the blood than in the lymph. Similarly, Endicott(2) recovered negligible amounts of orally administered radioactive iron in the lymph. These studies certainly seem to relegate the lymphatics to a minor role in iron absorption. It is conceivable that the iron stores of the reticuloendothelial system and the intestine(6) are in equilibrium with both the blood and interstitial fluid, in this case, lymph. The relative constancy of iron concentration in the lymph studied in these experiments is compatible with such an equilibrium as is the failure of iron concentration to keep pace with the rise in lymph volume produced by administration of 0.9% saline.

Blood plasma as the third possible source of lymph iron seems the most likely(1). The dialysis experiments point out a striking similarity between the adsorbed iron of lymph and that of plasma. From the work of Barkan(7), Laurell(8), Schade(9) and many others has evolved the concept that iron is present in plasma in combination with a specific globulin fraction, recently isolated and identified by Cohn(10). Because of this combination, serum iron is non-dialyzable at the pH of blood, but becomes dialyzable in more acid solutions(11).

Apparently a similar iron-protein linkage is present in lymph; and the protein resembles that of plasma in the range of pH over which it binds the iron firmly enough to prevent dialysis. Under the conditions of the experiments the protein is only about 60% saturated with iron.

Summary. The iron content of intestinal lymph of rats maintained on a normal diet is relatively constant over periods as long as seven days. At lymph outputs of one ml per hour, there is an hourly output of 0.5 γ of iron. With a 3- to 4-fold increase of lymph volume, iron concentration falls off and total output remains less than 1.0 γ per hour. The iron present in lymph is non-dialyzable at pH 7.0 to 4.7, but is dialyzable at pH 1.5. Added iron, up to concentrations of 48 γ per 100 ml in 2 specimens of pooled lymph, was non-dialyzable at pH 7, but all added iron in excess of this concentration was dialyzable.

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Received November 20, 1950. P.S.E.B.M., 1951, v76.

'Benemid' p-(Di-n-Propylsulfamyl)-Benzoic Acid: Lack of Effect of Aureomycin, Chloromycetin, Streptomycin and Terramycin.* (18442)

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Para-(Di-n-propylsulfamyl)-benzoic acid, 'Benemid',† increases by two to four times the penicillemia resulting from the administration of penicillin(1,2) and enhances the

* This study was made possible by a grant-in-aid from the Research Fund for Infectious Diseases of the University of Pennsylvania School of Medicine.

† Sharp & Dohme's trade-mark for p-(di-n-propylsulfamyl)-benzoic acid. The drug has been tentatively given the generic designation "probenecid".

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plasma concentrations of para-aminosalicylic acid (PAS)(3,4). The rapid elimination of penicillin from the body as contrasted to the much slower excretion of aureomycin, chloromycetin, streptomycin and terramycin indicates that these drugs are probably cleared by the kidneys by a different mechanism.

Because 'Benemid' has such a marked effect upon the plasma concentrations and the renal clearance of penicillin(5) by reason of its ability to suppress the tubular component of penicillin excretion, it was of interest to determine what effect, if any, the drug might have upon the plasma concentrations of aureomycin, chloromycetin, streptomycin and terramycin. Any effect on streptomycin would be of particular interest for streptomycin is administered in combination with PAS and 'Benemid' enhances the plasma concentrations of PAS. Since streptomycin has toxic properties, the incidence of toxic manifestations might be markedly increased if 'Benemid' interfered with the renal clearance of streptomycin. Should these circumstances obtain, it would be hazardous to employ 'Benemid' for its enhancement effect upon PAS when combined streptomycin and PAS therapy was being given. The following study was undertaken to clarify these points.

Subjects studied. Thirty-one individuals were studied; 6 individuals who received terramycin were healthy, adult male volunteers, the remaining 21 individuals were convalescent patients. None of the subjects of this investigation were suffering from apparent disorders of the liver, heart or kidneys.

Methods. Aureomycin and terramycin were assayed by the method described by Herrell and Heilman(6), which in this laboratory has checked well with that originally described by Dornbush(7) for aureomycin. Chloromycetin was assayed by the method of Smith and his associates(8). Penicillin was assayed by a cup-plate diffusion method described by Burke(9) employing Sarcina-lutea strain P.C.I. 1001. Streptomycin was assayed by a modification of the method of the Food and Drug Administration(10). The study was carried out in 2 parts, one with and one without the administration of 'Benemid' in conjunction with the administration of single doses of the antibiotic agents. Part I: each patient received a single dose of the respective antibiotic and thereafter blood samples were obtained at 1½, 3 and 6 hours. At least 48 hours were allowed to elapse in order to permit elimination of the antibiotic agents, a precaution necessitated because of the prolonged periods over which plasma concentrations of aureomycin and terramycin can be demonstrated following single administrations. Part II; after 48 hours the same patients received 0.5 g of 'Benemid' at 6 hourly intervals for 4 doses and were then given a fifth dose of 0.5 g of 'Benemid' in combination with the antibiotic agent under test. Thereafter, blood specimens were drawn at intervals that matched the time schedule followed in Part I.

Results. The data are presented in Fig. 1 and it is apparent that 'Benemid' had no effect on the plasma concentrations of aureomycin, chloromycetin, terramycin and streptomycin. Three patients received single doses of 250 mg of aureomycin, 3 patients received single doses of 1 g of chloromycetin, and 1 individual received 1 g of streptomycin with and without 'Benemid' and these patients showed the same lack of effect of 'Benemid'. (Table I).

In Fig. 2 are presented the average fractional 24-hour urinary recoveries of

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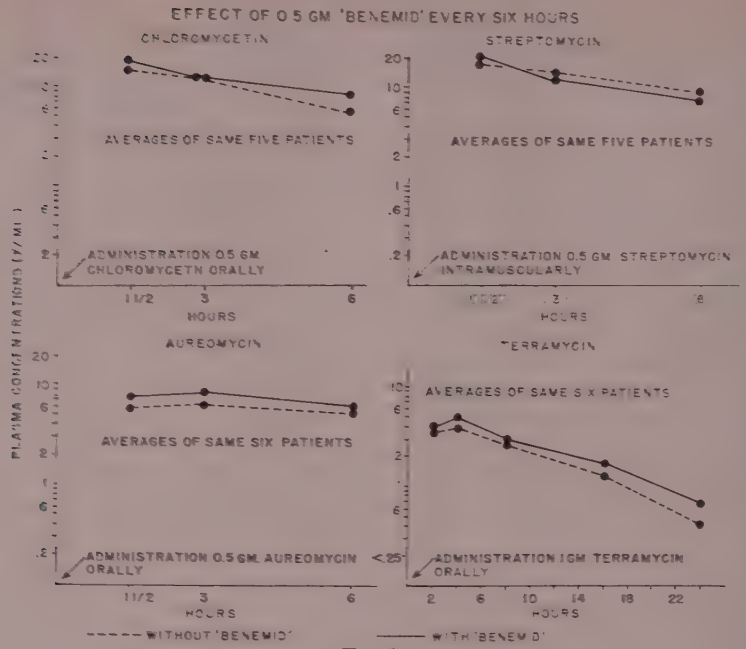

FIG. 1.

terramycin in six individuals. The recovery was 25% of the administered dose when terramycin was given either alone or in combination with 'Benemid'. The fractional urinary recoveries indicate that 'Benemid' not only did not quantitatively alter the recovery but did not influence the pattern of excretion of terramycin.

Discussion. Penicillin is cleared by the kidneys at a rate approximating renal plasma flow(11-13), and consequently penicillin disappears rapidly from the blood following single intramuscular injections of penicillin in aqueous solution. The rates at which aureomycin, chloromycetin, terramycin and streptomycin disappear from the plasma following the administration of single doses are much slower than for penicillin and differences in the renal elimination of these antibiotic agents are implied. It has been reported that the plasma concentrations of aureomycin were enhanced by the administration of carinamide(14), but this observation has not been confirmed in our hands. Carinamide and 'Benemid' are thought to act in the same manner(15), and as here reported 'Benemid' exerts no significant influence on the plasma concentrations of aureomycin. 'Benemid' plasma concentrations were determined on the specimens drawn for assay of antibiotic content and in all patients the 'Benemid' concentrations ranged between 5 and 10 mg per 100 ml of plasma, concentrations that have, without exception, increased penicillin plasma concentrations by 2 to 4 times(2).

Conclusion. Nine patients who received aureomycin, 9 patients who received chloramphenicol, 7 patients who received streptomycin and 6 patients who received terramycin failed to show any influence of p-(di-n-propylsulfamyl)-benzoic acid, 'Benemid', ad-

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TABLE I. Plasma Concentrations of Aureomycin, Chloromycetin, and Streptomycin With and Without 'Benemid'.

Patient	Age	'Benemid'*	Plasma conc.—meg per ml (hr after dose) 1½	3	6
		Aureomycin—.25 g			
E	49	0	2	4	2
		+	4	2	2
A	36	0	2	2	2
		+	4	4	4
F	37	0	2	3	4
		+	4	4	4
		Streptomycin—1 g			
JP	22	0	26.6	21.5	7.3
		+	25	23.8	10

Chloromycetin—1 g

Patient	Age	'Benemid'	Plasma conc.—meg/ml (hr after dose) ½	1½	2	3	4	6
S	69	0	7.5	15		7.5		
		+	7.5	15		22.6		
J	39	0	22.6		30		30	15
		+	30		30		30	22.6
JF	22	0	30		30		22.6	15
		+	30		45		45	30

ministered orally every 6 hours in a dose of 0.5 g on the plasma concentrations of the respective antibiotic agents. The failure of 'Benemid' to increase the plasma concentrations of streptomycin indicates that 'Benemid' may be administered safely to patients who are receiving streptomycin in conjunction with PAS.

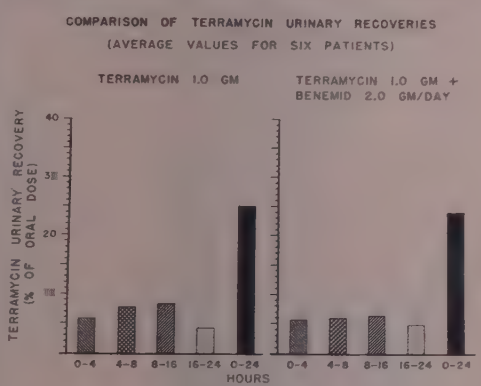

FIG. 2.

The authors are indebted to Heyden Chemical Co. for streptomycin, Lederle Laboratories Division of American Cyanamid Co. for aureomycin, to Parke, Davis and Co. for chloramphenicol, to Charles Pfizer and Co. for 'Terramcyin', and to Sharp and Dohme, Inc. for 'Benemid' employed in this study.

Received October 24, 1950. P.S.E.B.M., 1951, v76.

Eosinophile Levels in Hospitalized Psychotics During Combined Testosterone-Estrogen Therapy. (18443)

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In previous communications, the possibility that some forms of psychosis are caused by an operative or relative excess‡ of oxycorticosteroid was postulated(1,2) and the results of therapy with massive combined doses of testosterone and estradiol were reported (3,4). These results were improvement in 23 (58%) and the attainment of convalescent status in 12 (30%) in a series of 40 hospitalized psychotics.

As an index of adrenocortical activity, eosinophile levels were followed before and during the course of therapy since 11-oxysteroid secretion is reported to be reflected more sharply and consistently by the change in circulating eosinophiles than by the absolute lymphocyte count(5).

Materials and method. 17 patients chosen

TABLE I. Correlation of Improvement with DEC "Prognostic" Index.

Sex	Age	Diagnosis	Total dosage T/E, mg	Results T/E therapy	DEC index
F	20	S.-c.	1300/43.3	++++	36.7
F	30	S.	1100/36.6	++++	25.0
F	23	S.-s.	800/26.6	++	14.4
M	53	M.D.-d.	1450/76.6	++	13.6
F	56	M.D.-d.	800/26.6	++++	11.0
M	34	S.-p.	1250/41.8	++++	10.2
F	51	M.D.-d. inv.	487/31.6	++++	7.1
F	29	S.-p.	1100/36.6	0	5.9
M	46	S.-p.	1250/41.7	0	5.2
M	62	M.D.-m.	1300/43.3	0	5.1
F	17	S.-p.	750/33.2	(++++)*	3.1
F	30	S.-c.	1125/33.2	+	3.1
M	56	M.D.-m.	1200/36.6	(++)*	2.9
F	34	S.	900/31.6	+	2.7
M	53	I.M.-p.	1150/36.3	0	1.7
F	53	I.M.-p.	750/38.3	0	1.4
F	47	I.M.	1050/61.6	0	0.92

* Transient.

S.—Schizophrenia.

S.-s.—Schizophrenia, simple type.

S.-c.—Schizophrenia, catatonic type.

S.-p.—Schizophrenia, paranoid type.

M.D.-d.—Manic-depressive, depressed.

M.D.-m.—Manic-depressive, manic.

M.D.-d.inv.—Manic-depressive, depressed, involutional.

I.M.—Involutional melancholia.

I.M.-p.—Involutional melancholia, paranoid.

++++ = convalescent status; +, ++, +++ = improvement; 0 = unimproved.

DEC Index—Ratio of peak resting eosinophile level during or after course of treatment to average of resting levels before treatment.

Compiled in part from Data *in press*, Sackler *et al.* Sex Steroid Therapy in Psychiatric Disorders. Part I: Clinical Findings. *Acta Psych. et Neurol.*

* Deceased.

‡ By operative or relative excess is meant that relative concentration that produces an effect as distinguished from the absolute concentration.

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at random from a series of 40 were subjected to weekly eosinophile determinations before, during and after a course of combined sex steroid therapy. The basic treatment procedure consisted of administration of 50 to 100 mg of testosterone propionate and 1.67 to 3.33 mg of estradiol benzoate in a single intragluteal injection administered daily for a period of 4 weeks. Fasting blood was obtained by finger puncture and direct counts were promptly performed according to the method described by Thorn(6), employing the Levy 0.2 mm deep chamber and eosin-acetone diluting fluid. The average of 4 chamber counts was computed for each determination (Table I).

Results. 1. The pre-treatment eosinophile levels fluctuated erratically during the several weeks they were followed.

2. The eosinophile counts of all but 3 patients rose sharply during the course of testosterone-estrogen therapy, as may be seen in Fig. 1. Reaching a peak usually in the latter 2 weeks of the 4-week period of treatments, they increased as much as 30-fold before reverting to lower levels 4 to 5 weeks after therapy.

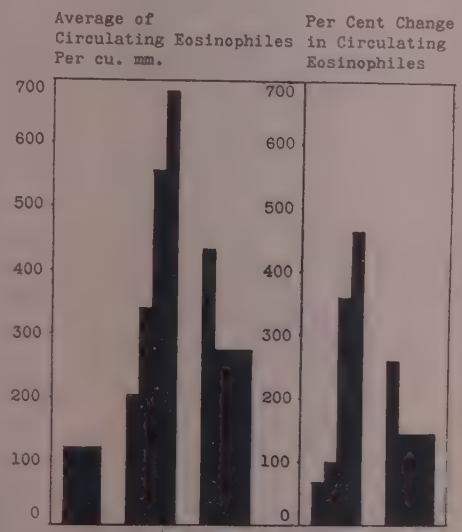

FIG. 1.

Eosinophile levels of 17 patients before, during, and after testosterone-estrogen therapy.

3. There was positive correlation between improvement in the patients' clinical status and the attainment of higher eosinophile levels, as demonstrated in Fig. 2.

Discussion. In our recent report on "Some Common Physiologic Denominators of Histamine, Sex Steroids, Insulin and Electroconvulsive Therapies"(7), the effect of these agents on adrenocortical activity was presented. It was considered diphasic—stimulation initially (by all but sex steroids), and then depression (by all these therapies, including sex steroid). In many of the histamine treated patients we have noted a slow but steady rise in the eosinophile level. Courses of insulin coma and electroconvulsive therapies have also been reported(8-11) to be associated with a rise in eosinophile or lymphocyte resting level.

Finn Rud(7) considers a rise in the eosinophile count a favorable prognostic sign in psychosis: "In many cases a close relationship to the clinical course was shown, the eosinophile count increasing during improvement of the psychic condition." Rud also followed the eosinophile levels of psychotic patients during insulin shock therapy. While he found a characteristic *decrease* during the first few hours after injection of each dose of insulin, during extended treatment there was a distinct *increase* in the resting level of eosinophiles, often of several hundred per-cent. This increase first appeared in the second or third week and gradually reverted to the original level after the conclusion of

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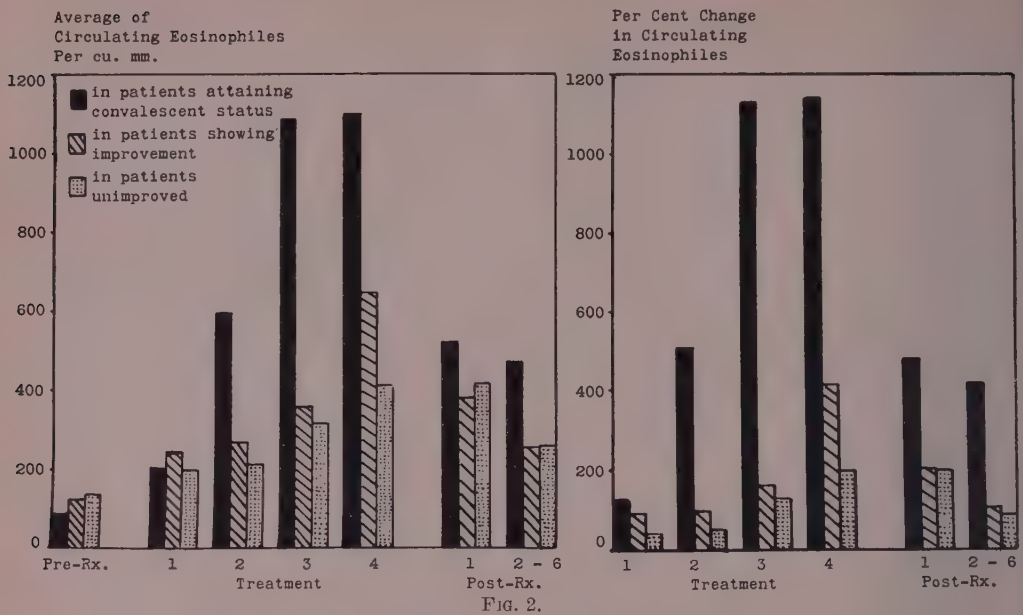

FIG. 2.

Correlation between clinical improvement and change in circulating eosinophile levels of 17 patients: 6 attained convalescent status, 5 improved, and 6 unimproved.

therapy. This may be interpreted as representing a "washing out" of adrenocortical hormone during the acute stress of shock treatment, with eventual attainment of lower basal levels of secretion of 11-oxysteroids as reflected in the higher eosinophile counts.

There is evidence that the sex steroids may have a direct antagonistic effect on corticosteroids. Venning and Brown(12), Talbot *et al.*(13) and Albright(14) have each reported a marked fall in 11-oxycorticosteroid excretion in patients following the administration of testosterone propionate. The latter also reports that Dr. Edward Dempsey has found decreased steroids in the cortices of rats as well as in biopsies from humans following testosterone administration. The eosinophile changes here reported tend to support their observations.

Summary and conclusions. 1. The direct eosinophile counts of the majority of a group of 17 psychotic patients rose sharply during the course of massive combined testosterone-estrogen therapy. 2. There was an apparent positive correlation between improvement in the patients' clinical status and the degree of eosinophilia attained. 3. An anti-adrenocortical effect at least in relation to blood eosinophile levels of sex steroids is suggested.

Authors gratefully acknowledge encouragement and assistance of Drs. H. A. LaBurt, Creedmoor State Hospital, Sydney S. Lazarus, Felix Marti-Ibanez and Marvin Weinberg. The work was carried out with the enthusiastic participation of Mrs. Ann Nichol. The expenses of this study were defrayed by a grant made in memory of Isaac Sackler.

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Received November 15, 1950. P.S.E.B.M., 1951, v76.

Retention and Turnover of Radiocalcium by the Skeleton of Large Rats.* (18444)

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Radioactive calcium has been employed (1-6) under various conditions in studies of calcium metabolism in the rat. Armstrong and Barnum(5) presented evidence that some of the different anatomical forms of the calcified tissues exchange calcium at unequal rates. Norris and Kisieleski reported(6) that the femora and scapulae of rats given radiocalcium by intravenous injection reached their maximum content of the radioelement in approximately 100 minutes after the injection and showed no significant decrease in the amount retained in 6 to 8 days.

This investigation was conducted to obtain data as to the turnover of calcium in selected calcified tissues of the rat over an extended period of time. A second purpose was to determine the fraction of the dose of radiocalcium retained in the animal at various times after the administration of the radioisotope. For these purposes radiocalcium (Ca^{45}) was administered to rats and the animals were sacrificed at intervals following the administration of the isotope.

Experimental. Thirty-six male rats of the Sprague-Dawley strain of comparable age whose weights lay between 250 and 269 g, averaging 265 g, were given intraperitoneal injections of a high specific activity† radiocalcium solution. Each rat received 3 injections of 2 ml of a solution of the isotope in isotonic saline at 2 hour intervals. The average dose of radiocalcium per animal was approximately 8.3 x 10^5 counts per minute, as determined by our methods of sample preparation and of radioactivity measurement. The animals were sacrificed in pairs at intervals varying from 1 to 180 days after the injections with the exception that at 43 days and at 170 days 5 animals were killed. Twenty-four hours before sacrifice the animals were placed in metabolism cages and the urine collected. On 5 occasions fecal collections were made at the same time. While in the metabolism cages the animals were fed a diet devoid of calcium in order to eliminate contamination of the excreta with the calcium of spilled food and to reduce the amount of unabsorbed food calcium in the alimentary tract. The femora, humeri, the 3 lower lumbar vertebrae and teeth were obtained by dissection and freed of soft tissues, a process which was facilitated by boiling the specimens in water. Both epiphyseal ends of the femurs were cut off using a rotating dental disk through recognizable anatomical landmarks. The marrow was removed from the femur diaphyses by scraping and by use of a pledget of cotton on a probe. The 4 epiphyseal ends of the femurs of a single animal were pooled as one sample and the two diaphyses were united as another sample. The humeri were combined as one sample as were the vertebrae. The pooled molar teeth and the pooled incisor teeth were treated as separate samples. The remainder of the animal not present in the above named calcified tissues constituted the carcass residue.‡ The carcass residues of the first 28 animals to be sacrificed were heated

* This study was supported by a grant from the Research Grants Division of the U. S. Public Health Service.

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† Specific activity 80 mc/g Ca. The radiocalcium was obtained from The Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

in 200 ml of strong sodium hydroxide solution until disintegration of the soft tissues had been effected. The mixture was then converted into a uniform soap-like suspension in a Waring blendor, a process which reduced the bones to fine fragments, and 2 large weighed aliquots of the suspension were taken for analysis. The carcass residues from the last 8 animals to be sacrificed were wet ashed in concentrated nitric acid(7) and diluted to an appropriate volume. All other samples, including the urine and feces, were dried, ashed at 700°C, dissolved in hydrochloric acid and diluted to known volumes. Some samples of feces ash required fusion with sodium carbonate before solution in hydrochloric acid.

Total calcium was determined by the method of Clark and Collip(8). The calcium in appropriate aliquots of the solutions was precipitated as calcium oxalate monohydrate at pH 5(9). The precipitates were collected as "infinitely thick" samples§ using the apparatus of Armstrong and Schubert(10) and the radioactivity measurements were made with a thin mica window Geiger-Mueller counter. All measurements for radioactivity were corrected for background, resolving time losses, radioactive decay and for changes in counter efficiency. The reproducibility of the radioactive assays is indicated by the results obtained with duplicate aliquots of the carcass residues which agreed within the statistical variation of the count, namely 1.5%. The total calcium content and the fraction of the administered radiocalcium remaining in each animal were calculated from the sum of the results obtained with the carcass residue and the separately examined tissues. The specific activity of each sample describes the results of the division of the percentage of the injected dose of radiocalcium found in the sample by its total calcium content expressed in milligrams.

Results and Discussion. The average result obtained with each of the tissues and with the urine of the animals sacrificed at each time interval is presented in graphic form. The calcium specific activities of the femoral diaphyses, femoral epiphyses, humeri, lumbar vertebrae and total skeletons were considerably different on day 1, but decreased with time to nearly identical values on the 52nd day (Fig. 1). The finding that, in 52 days, there was effected a virtual equality of distribution of the radioisotope in the calcium of the separately examined bones, and within the skeleton as a whole, shows that an intraskeletal redistribution of the exchangeable calcium occurs in the same period.

After the 52nd day the specific activities of the calcified tissues decreased at a much reduced rate (Fig. 1). The decline in specific

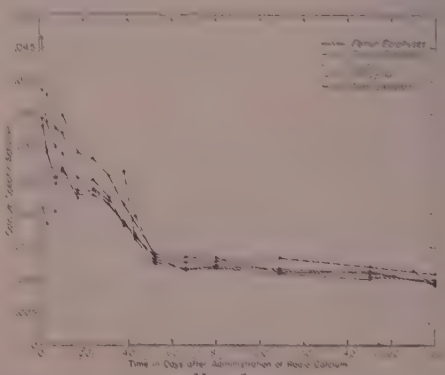

FIG. 1.

Changes in calcium specific activity of selected bones and of whole skeleton of rats following administration of radioactive calcium.

‡ Since the calcium content of extra- and intracellular fluids is very low in comparison with that of the skeleton the calcium of the carcass residues can be considered to have been mainly situated in the bones other than those selected for separate examination.

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§ It has been found in this laboratory that an infinitely thick sample for Ca^{45} beta radiation is 50 mg/cm^2. Inert calcium was added, before precipitating calcium oxalate, in those cases in which the amount of this element contained in the aliquot of the solution of the tissue was insufficient to give an infinitely thick layer of calcium oxalate.

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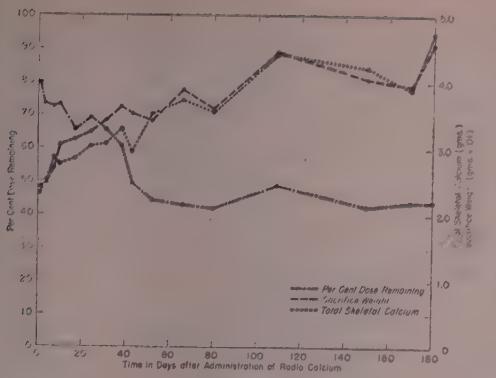

FIG. 2.

Body weight growth and accretion of skeletal calcium in rats. Retention of radiocalcium by rats following intraperitoneal administration.

activities which did occur after the 52nd day was not due, in any important degree, to loss of radiocalcium from the skeleton by excretion since, from the 52nd day onwards, the fraction of the administered dose retained in the animals was maintained very nearly constant at about 42-45% (Fig. 2). Radiocalcium was found in all samples of excreta examined. However, the amount present in 24-hour samples of urine on 8 occasions between the 50th and 180th days averaged only 0.006% and never exceeded 0.013% of the injected dose of radiocalcium. Each of 6 one-day fecal collections made after the 42nd day contained 0.02-0.03% of the administered radioisotope. The reduction in specific activity of the skeleton and its components which occurred between the 52nd and 180th days was due mainly to dilution of the radiocalcium, which remained nearly constant in amount, with the normal isotope of calcium gained by the skeleton as it grew. The decrease in measured specific activity of the whole skeleton after the 52nd day was 26.0% (from 0.0125 to 0.0093) as shown in Fig. 1. In the same time the amount of calcium which accrued to the animals (Fig. 2) was 25.8% of the quantity present at 180 days. The same line of reasoning leads to the conclusion that a part of the rapid reduction in skeletal specific activities occurring before day 52 was also due to bone growth since, as seen in Fig. 2, 34.1% of the skeletal calcium present at day 52 was acquired after day 1. It can be calculated, from this figure and from the fact that 44.4% of the Ca^{45} present on day 1 was excreted by day 52 (Fig. 2), that 29.9% of the measured reduction in skeletal specific activity occurring between days 1 and 52 was due to skeletal growth and the remainder to excretion of radiocalcium. These considerations emphasize the importance of taking into account changes in amount of a tissue constituent, as may occur in a relatively short time in rats by growth, in studies of the turnover of the constituent with tracer isotopes.

The observations reported in this paper indicate that the radiocalcium was deposited in the bones of the animals in forms which differed greatly in their relative abilities to permit mobilization and excretion of the labelled calcium. These forms of incorporation of radiocalcium and the parts of the skeletal calcium associated with each may be referred to as the mobile fraction and the fixed fraction. The fixed radiocalcium is that fraction, amounting to *circa* 45% of the injected dose, which was maintained with only a slight excretion after the 52nd day. It is suggested that the fixed radiocalcium was that which eventually became incarcerated by bone growth, or by rearrangement of the calcium atoms in the bone salt, in positions so remote from the body fluids as greatly to interfere with its mobilization and excretion. Since the specific activity of the several bone types was maintained at quite similar values after the 52nd day (Fig. 1), it can be concluded that equal weights of bone calcium had, by the 52nd day, incorporated fairly uniform amounts of radiocalcium in the fixed form and that this relationship was not disturbed by further bone growth. The amount of radioisotope deposited per unit weight of bone calcium differed originally among the bone types. However, by the 52nd day all of the easily mobile or exchangeable radiocalcium had been excreted‖ or converted into the fixed form, thus reducing the specific activities of all bones to uniform values. After the 52nd day the excretion of radiocalcium occurred only from the difficultly mobilizable bone calcium. The

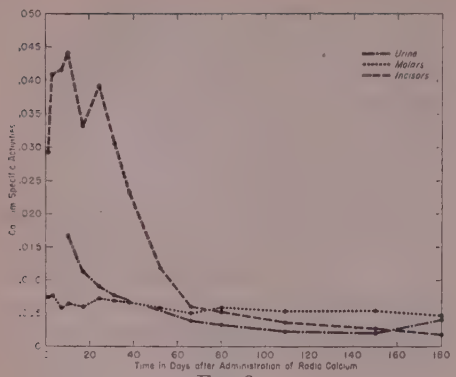

FIG. 3.

Changes in calcium specific activity of teeth and urine of rats following administration of radiocalcium.

results obtained from the urine examinations indicate that this form of bone calcium was excreted at a constant low rate.

Results as to the specific activity of the urine specimens collected before day 10 and on day 38 are not reported because of errors in the determinations of total calcium due in some cases to the very low calcium content of the urine specimens. However, radiocalcium assays demonstrated the presence of an average of 4% of the injected dose in the urine secreted on day 1 and that the average amount in the daily urine rapidly decreased to 0.032% on day 3 and to 0.015% on day 10. From the 66th day onwards the specific activity of the urine was maintained essentially unchanged at a value approximately one-fourth that of the skeleton (Figs. 1 and 3). Since the calcium of the urine is derived from that of the plasma it can be assumed that the specific activity of the calcium of the body fluids, including the saliva, paralleled that of the urine(11).

The specific activities of the molar teeth were low and showed a gradual decline to a value which on day 180 was about 60% of that on day 1 (Fig. 3). It is probable that these non-growing organs acquired some part of their radiocalcium by exchange of calcium between the enamel and the saliva when the saliva had a very high specific activity immediately after the injections. The incisor teeth of rats are known to be worn away at the incisal edge and to be renewed by growth at the root end at approximately 50 day periods(12). On this account the incisor teeth acquired much more radiocalcium than the molar teeth during the period of high specific activity of the calcium of the body fluids. The radiocalcium fixed in the incisor teeth was mainly lost by attrition as is shown by the fact that their specific activities rapidly declined from the 10th to the 66th day, a period of time which is in agreement with the expected time for the replacement of the teeth. After the 66th day the specific activity of the incisor teeth, as they continued to be formed, was maintained very nearly equal to that of the urine and hence to that of the plasma.

As shown in Fig. 2, the calcium content of rats, exclusive of the teeth, is close to 1% of the body weight in animals weighing between 249 and 465 g.

Summary. The calcium specific activities of the femoral epiphyses, femoral diaphyses, humeri, lumbar vertebrae and the remainder of the skeleton of large rats differ soon after the administration of radiocalcium but decrease to a common value after 52 days, following which the specific activities decline at equal but much reduced rates. The fraction of the injected dose of radiocalcium retained in the animals declines to 42-45% on the 52nd day, after which only small quantities of radiocalcium are excreted. The results indicate the existence in large rats of two kinds of skeletal calcium which differ markedly in their rate of turnover and in the length of time over which they fix radiocalcium. The apparent reduction in skeletal calcium specific activity occurring in large rats after the 52nd day following the injection of radiocalcium is due to accretion of calcium to the

‖ The radiocalcium content of the freely exchangeable bone fractions would not be expected to be zero after the 52nd day but would correspond to the specific activity of the body fluids. There is evidence (vide infra) that the latter specific activity was maintained, at least from the 66th day onwards, at a low and uniform value.

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skeleton. Part of the decline in skeletal specific activities which takes place in rats before radiocalcium excretion ceases is also due to skeletal growth.

Data as to the changes of calcium specific activity of the urine and the teeth following the injection of labelled calcium are presented and discussed.

Received November 27, 1950. P.S.E.B.M., 1951, v76.

Toxicity of Glycine for Vitamin B_{12}-Deficient Chicks.*† (18445)

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An unidentified factor which was reported to stimulate growth of chicks fed a vegetable protein diet also lowered the blood non-protein nitrogen level(1). It was later found(2) that a vit. B_{12} concentrate lowered the blood non-protein nitrogen of chicks previously fed a vit. B_{12}-deficient diet. Non-protein nitrogen and urea were found to be higher in zoopherin (vit. B_{12})-deficient rats than in normal rats (3). These and other reports(4,5) indicated that vit. B_{12} is concerned with nitrogen utilization and metabolism. It was the purpose of this experiment to investigate further the vit. B_{12}-nitrogen relationship by feeding chicks a known amount of non-protein nitrogen and observing the effect of vit. B_{12} deficiency.

Procedure. Twenty-eight day-old White Leghorn chicks which had been fed the vit. B_{12}-deficient diet shown in Table I from the day of hatching were used in this experiment. A deficient control group received no treatment, whereas 3 μg of vit. B_{12} per week were injected subcutaneously into the chicks of the supplemented group. The night of the 28th day food was withdrawn from chicks in both groups. The following morning blood was taken from 4 birds of each group by heart puncture, and the birds sacrificed. Protein-free filtrates were prepared from pooled samples of the plasma by tungstic acid precipitation. The filtrates were analyzed for nitrogen by a semi-micro Kjeldahl method and for amino nitrogen using the naphthoquinone-sulfonic acid method(6). Immedi-

TABLE I. Experimental Diet.

	%
Ground yellow corn	43.5
Ground wheat	15
Soybean oil meal (solvent)	37
Dehydrated alfalfa	1
Ground limestone	1.5
Dicalcium phosphate	1.5
Sodium chloride	.5
A and D supplement*	.1
Choline chloride	.1
Methionine	.1
$MnSO_4$	8 g/100 lbs.
	mg per lb.
Riboflavin	4.5
Ca pantothenate	7.5
Niacin	15.0
Thiamin	2.5
Pyridoxine	2.5
Biotin	0.045
Folic acid	0.45
Inositol	4.53
Menadione	4.5
Alpha tocopherol	4.5
Para amino benzoic acid	2.5

* 5,000 I.U. vit. A and 1,000 AOAC chick units vit. D per g.

* This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative 171.

† Published as scientific paper 964, Washington Agric. Exp. Stations, Inst. of Agric. Sciences, State College of Washington, Pullman.

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TABLE II. Results of Pooled Blood Plasma Analyses.

Time after glycine dose, hr	B_{12} deficient		B_{12} injected	
	N.P.N., mg %	Amino N, mg %	N.P.N., mg %	Amino N, mg %
0	24	9	—	8
2	20	19	35	20
4	43	20	32	16

ately after this bleeding, capsules containing 1 g of glycine were force-fed to all remaining chicks of both groups. Two and 4 hours after the glycine feeding blood samples were obtained by heart puncture and treated as above. Segments of liver and kidney were taken for histological examination. These were fixed in Carnoy's solution and stained with hematoxylin and eosin.

Results. Two hours after the glycine administration, the birds of both the deficient and supplemented groups seemed normal with respect to appearance and behavior. Four hours after the glycine dosage, a dramatic change appeared in the vit. B_{12}-deficient chicks. Two had died and those which remained alive were extremely weak and semicomatose. Several were unable to stand and appeared near death. It was extremely difficult to obtain blood from these birds, probably because of circulatory failure. In marked contrast, the vit. B_{12}-treated chicks appeared normal and lively.

Microscopic examination of histological sections of liver and kidney tissue revealed no difference between the two groups. The results of the blood plasma analyses are shown in Table II.

In a second trial a similar procedure was followed except that food was left before the chicks at all times. Four hours after glycine feeding, the chicks of the vit. B_{12}-deficient group were noticeably listless although they were only slightly affected when compared with the starved B_{12}-deficient chicks of the first trial. The B_{12}-injected birds again seemed normal. No blood or histological examinations were performed on these birds.

Discussion. Both the plasma non-protein nitrogen and amino nitrogen levels showed that the vit. B_{12}-injected birds were able to metabolize the administered glycine better than the vit. B_{12}-deficient birds. In the former group, NPN and amino N were highest 2 hours after glycine administration and had diminished by 4 hours afterwards. In the B_{12}-deficient group, however, NPN and amino N levels did not fall after 2 hours but increased to some extent at 4 hours. These high levels were associated with the toxic effect of the glycine. It was interesting to note that vit. B_{12}-deficient chicks which were on feed during the whole period were not as affected by the glycine as those which were starved. It is possible that energy as well as vit. B_{12} is required in nitrogen metabolism.

Summary. The plasma levels for non-protein nitrogen and amino nitrogen after administration of glycine were higher in vit. B_{12}-deficient chicks than in B_{12}-injected chicks. One gram of glycine, when force fed in gelatin capsules, was toxic to starved, B_{12}-deficient chicks and less toxic to B_{12}-deficient chicks which had not been starved. Chicks which had been injected with vit. B_{12} were able to withstand the glycine whether or not they had been without food.

Received November 27, 1950. P.S.E.B.M., 1951, v76.

Antagonistic Effect of Serum on Bacteriostatic Action of Lupulone. (18446)

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The loss of the antibiotic properties of lupulone when mixed with serum has been noted by Salle, Jann, and Ordanik(1) and by Chin, Chang, and Anderson(2). An effort has been made by this laboratory to determine the cause of this inactivation in order that the

TABLE I. Inhibition Zones of Lupulone Mixed with Serum Fractions.

Fraction added	15%* serum equivalent		5%* serum equivalent	
	B. subtilis	C7	*B. subtilis*	C7
—	21.7 mm	21.9 mm	21.7 mm	21.9 mm
I	13.7	13.2	15.7	15.2
II + III	<10	<10	10.5	<10
IV	<10	<10	<10	<10
V	<10	<10	11.2	15.5
VI	17.6	20	18.5	19
Whole plasma	<10	—	<10	<10
Whole serum	<10	<10	<10	<10

* Total solids basis.

limits of usefulness of this antibiotic(3) might be better realized.

Materials and methods. Cup plate assays were performed on Medium II* agar, with *Bacillus subtilis and Micrococcus sp* (C7) serving as test organisms. Manometric oxygen uptake experiments were carried out in 0.04 *M* phosphate buffer, pH 7, containing 0.01 *M* succinate as substrate. A suspension of *B. subtilis* grown 18 hours on Medium II at 35°C in a shaking machine served as the test organism and was added from the side-arm. The cells were washed once in 0.025 *M* phosphate, pH 7, and resuspended in buffer of the same composition. Growth experiments were carried out as follows. Tubes containing 10 ml of medium IIb† and varying amounts of lupulone were supplemented with 0.5 ml of normal or extracted plasma. All tubes were then inoculated with one drop of an 18-hour broth culture of *Streptococcus fecalis* grown on medium IIb. Turbidity was determined periodically with a Coleman Model II spectrophotometer, at 650 mμ.

Aqueous lupulone suspensions were generally prepared from concentrated alcoholic solutions of crystalline lupulone. Lecithin and cephalin preparations from eggs were obtained from E. B. Kester of this laboratory. Citrated plasma, supplied through the courtesy of Cutter Laboratories. was used rather than serum in most experiments. It had lupulone-inactivating properties equivalent to fresh serum. Serum fractions were kindly supplied by E. J. Cohn of the Physical Chemistry Department of Harvard University.

Experimental. In order to find, if possible, a particular serum component which had the capacity to antagonize lupulone antibiosis in a high degree, serum fractions were tested for this property. The fractions were dissolved in water to levels of 3.75 mg/ml and 11.25 mg/ml, which are approximately equivalent to 5% serum and 15% serum, respectively, on a total-solids basis. Five ppm of lupulone was added to the solutions, and cup assays run on the lupulone-serum fraction mixtures. Results are shown in Table I. Fractions II + III and IV have a considerable capacity to inactivate lupulone, whereas Fractions I and VI have only feeble inactivating powers. The description of these fractions(4) suggests that the lipoidal constituents or β-globulin might very likely be associated with the lupulone-inactivating properties, while albumin and fibrinogen are apparently eliminated as possibilities. Manometric experiments yielded essentially the same results as those shown in Table I. To test the importance of lipids in lupulone inactivation, Fraction IV was extracted for 24 hours with ether and the residue

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3. Lewis, J. C., Alderton, G., Carson, J. F., Reynolds, D. M., and Maclay, W. D., *J. Clin. Invest.*, 1949, v28, 916.

* Peptone, .5%; yeast extract, .15%; beef extract, .15%; dextrose, .1%; KH_2PO_4, .39%; NaCl, .35%; pH 6.8 with NaOH.

† Yeast extract, .50%; N-Z Amine, .2%; dextrose, .1%; NaCl, .35%; KH_2PO_4, .39%; pH, 6.8 with KOH.

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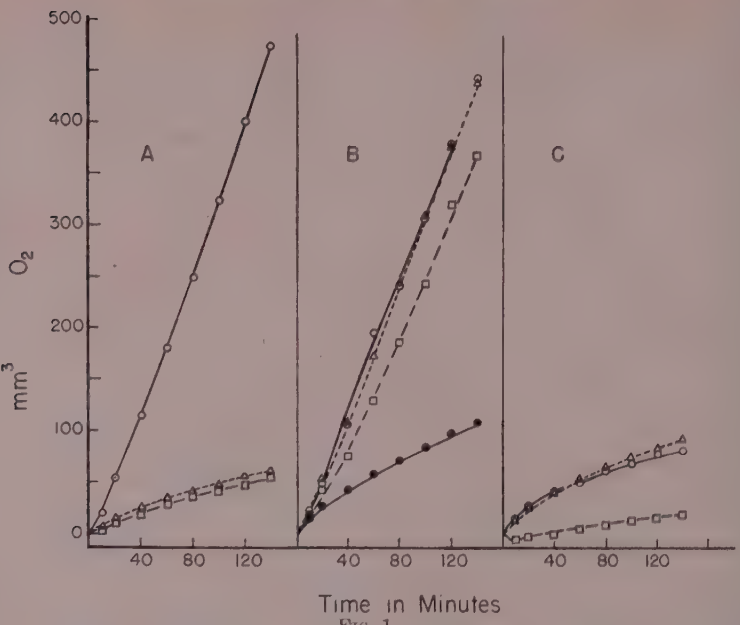

FIG. 1.

Influence of lecithin on the action of lupulone on the respiration of *B. subtilis*. A. Inhibitory action of lupulone on respiration of *B. subtilis*. △ endogenous respiration, ○ succinate substrate, □ succinate + lupulone. B. Influence of lecithin on inhibitory action of lupulone. ○ lecithin (.05%) + succinate, △ lecithin (.05%) + succinate + lupulone, □ lecithin (.01%) + succinate + lupulone, ● lecithin (.05%). C. Additional controls: △ lecithin (.05%) + lupulone, ○ lecithin (.01%) + lupulone, □ lecithin (.05%) + succinate + lupulone (cells heat killed). Lupulone concentration is 4 ppm in all cases.

tested manometrically. Its lupulone-inactivating capacity was found to be essentially unimpaired. Fraction IV was also autoclaved for 15 minutes at 15 lb pressure, and then dialyzed for 24 hours against running water. The non-dialyzable residue was still highly active. If, however, this autoclaved, dialyzed material were extracted with ether for 24 hours, practically all of its lupulone-inactivating properties were removed, while the ether extract possessed marked capacity to inactivate lupulone. These results suggested that lipoidal materials, probably bound to proteins, might be implicated.

To test this hypothesis, lecithin and cephalin prepared from eggs were tested manometrically and found highly effective in reducing lupulone inhibition, as was fresh egg yolk. The following compounds were tested and found void of activity: cholesterol, egg albumin, yeast autolysate, NZ-case, and choline, as well as lauric, myristic, and palmitic acids. The influence of varying quantities of lecithin on the inhibition of respiration of *B. subtilis* by lupulone is shown in Fig. 1.

That lecithin was allowing succinate to be metabolized, and not merely providing the cells with a substrate oxidizable via a resistant metabolic pathway, was demonstrated by omitting succinate in parallel cups. It is apparent that oxygen uptake is markedly greater in the presence of succinate. The slow acceleration of oxygen uptake at the lowest phosphatide concentration suggests that lupulone inhibition is neutralized more slowly, but that cells are still capable of metabolism once the lupulone has been "inactivated"; *i.e.*, lupulone poisoning is reversible. This was more clearly demonstrated by adding plasma from a second side-arm of the Warburg vessels after various periods of lupulone inhibition. It was found that lupulone inhibition was completely reversible, even

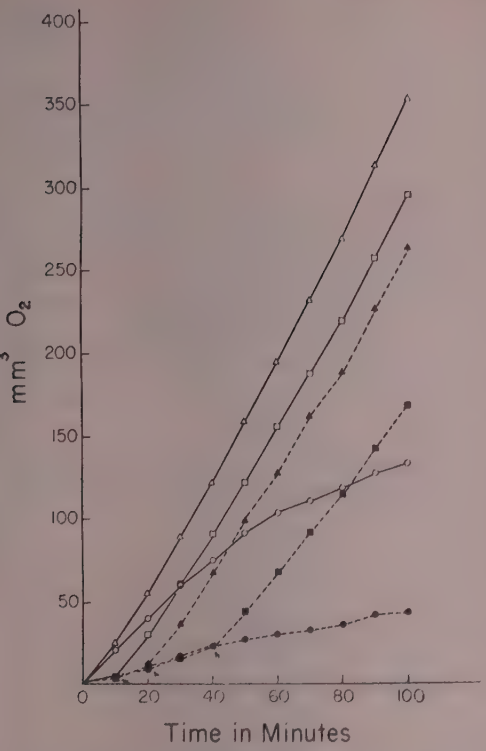

FIG. 2.

Reversibility of lupulone inhibition by the subsequent addition of plasma. ● Oxidation of succinate in the presence of lupulone. △ Plasma added at 0 minutes, □, ▲, ■ plasma added at time indicated by arrow, ○ oxidation of plasma in the presence of lupulone (succinate omitted). Final concentration of plasma in each vessel is 2.5%. Lupulone concentration is 4 ppm.

after 40 minutes (See Fig. 2).

In an effort to demonstrate more convincingly that the phospholipids are responsible for the lupulone-inactivating properties of plasma, we attempted to remove them from whole plasma. Conventional methods for the extraction of phospholipids generally involve treatment with alcohol-ether, which renders the proteins insoluble and thus makes impossible further evaluation of the residue. However, we were able to remove considerable amounts of lipid phosphorus by emulsifying plasma with two volumes of chloroform, freezing at —30°C, and discarding the chloroform fraction. By repeating this process six times, it was possible to extract 31% of the total P, or about 45% of the lipid phosphorus, as determined by the method of Schmidt *et al.* (5). The neutralizing capacity of this extracted plasma was then compared with that of normal plasma by the manometric technic. The results shown in Table II indicate that about half of the neutralizing capacity of the plasma had been lost. Controls lacking succinate, but containing lupulone and plasma, demonstrate that the extractable materials increase the oxidation of succinate in the presence of lupulone. These results were verified in a growth experiment, using *S. fecalis* as described earlier. The results shown in Table III clearly indicate a marked reduction in neutralizing capacity of the expected order of magnitude.

Discussion. Although the work presented here strongly indicates that the serum phosphatides can inactivate lupulone and that extraction of lipid phosphorus is closely correlated with loss of lupulone-inactivating ability, it must be emphasized that we have not obtained evidence demonstrating that the phospholipids are *solely* responsible for the antagonistic effect of serum on the antibiosis

TABLE II. Influence of Normal and Extracted Plasma* on Inhibition of Respiration of *B. subtilis* by Lupulone.

Cup contents (other than buffer, cells and KOH)	μl O_2 uptake at 120 min.
-	266
Succinate	885
Succinate + lupulone†	71
Succinate + lupulone† + normal plasma‡ (5%)	1004
Succinate + lupulone† + normal plasma‡ (2.5%)	915
Succinate + lupulone† + normal plasma‡ (1.67%)	660
Normal plasma (2.5%) + lupulone	436
Normal plasma (1.67%) + lupulone	374
Succinate + lupulone + extracted plasma (5%)	826
Succinate + lupulone + extracted plasma (2.5%)	576
Succinate + lupulone + extracted plasma (1.67%)	333
Extracted plasma (5%) + lupulone	558
Extracted plasma (2.5%) + lupulone	382

* Residue after 6 chloroform extractions.

† Lupulone concentration is 4 ppm in all vessels where employed.

‡ Figures in parentheses refer to concentration of plasma in vessel.

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TABLE III. Influence of Normal and Extracted Plasma* on Growth of *S. fecalis* in the Presence of Lupulone.

Lupulone	Total growth ($\log G_{initial} - \log G_{final}$) Normal plasma	Extracted plasma
—	.418	.421
5 ppm	.309	.284
10 ppm	.258	.194
20 ppm	.186	.046

* Residue after 6 chloroform extractions.

of lupulone. In fact, rough calculations comparing the antagonistic action of plasma with that of purified egg phosphatides indicate that the latter are only about one-tenth as active as might be anticipated. Substances in serum other than phospholipids may have considerable lupulone-inactivating property, or egg phosphatides may be less active than plasma phosphatides, or purified phosphatides may be less active than phosphatides occurring in the natural state (presumably as lipo-proteins).

The fact that lupulone inhibition was found to be reversible by the subsequent addition of plasma is evidence of the bacteriostatic nature of the lupulone antibiosis and contrasts with the irreversible action of detergents(6), where only prior or simultaneous addition of phosphatides is effective.

Summary. 1. Lecithin and cephalin are capable of neutralizing the inhibiting properties of lupulone. 2. Evidence is presented indicating that the antagonistic effect of serum on the bacteriostatic action of lupulone is due, at least in part, to its phosphatide content. 3. The inhibition of respiration by lupulone may be reversed by the subsequent addition of plasma.

The authors wish to thank Elizabeth A. McComb of this laboratory for the determinations of phosphorus.

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Received November 27, 1950. P.S.E.B.M., 1951, v76.

Variations in Anti-Hyaluronidase Titre of Normal Human Serum with Age and Sex.* (18447)

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In the course of studies on the serum anti-hyaluronidase in leukemia, and lymphosarcoma, by means of the viscosimetric method, it became necessary to reinvestigate the anti-hyaluronidase titres of normal human serum. Studies of the anti-hyaluronidase titre measured by the turbidimetric method, have shown that appreciable variations in the titre occur between certain age groups(1,2), and between sexes(2). However, none of the studies dealing with viscosity(3-7) have noted any variations in the titre due to sex, within the age groups studied. As the present data indicate, the anti-hyaluronidase titre in the serum of the normal female is appreciably higher than that of the normal male.

Method. The viscosity method as reported by Haas(3) has been used. The anti-hyaluronidase titre is expressed as the percent inhibition of the hyaluronidase activity by 0.02 ml of serum.

Results. The data are summarized in

* Aided by a grant from the Charles R. Blakely Fund of the National Research Council.

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TABLE I. Variations in the Anti-Hyaluronidase Titre with Age and Sex. Total No. cases, 72; males, 35; females, 37.

	Male				Female			
Age, yrs	No. cases	%	S.D.	$S.E._m$	No. cases	%	S.D.	$S.E._m$
0-20	2	21.2	—	—	1	—	—	—
21-30	9	31.2	5.09	1.78	10	32.8	4.16	1.39
31-40	9	24.9	4.00	1.40	8	34.7	8.88	3.36
41-50	6	31.1	5.37	2.40	10	39.8	6.94	2.32
51-60	6	28.7	4.77	2.14	5	39.4	3.64	1.82
61-80	3	34.9	—	—	4	37.5	—	—

Both sexes group 21-30 mean = 32.0% ± 0.997.

Male group, 31-60
Mean — 28.2% ± 1.220
S.D.—3.13
$S.E._m$—1.809%

Female group, 31-60
Mean — 38.0% ± 1.926
S.D.—2.84
$S.E._m$—1.749%

TABLE II. Comparison of Male and Female Titres.

Age, yrs	Sig. ratio	Level of sig., %
21-30	0.770	46.0
31-40	2.694	1.7
41-50	2.610	2.0
51-60	3.816	0.6

Table I. Between the ages of 21 and 30 years, the mean anti-hyaluronidase titre (percent inhibition) is the same. Beyond this age level, a significant difference in titres appears in the serum of the two sexes. The anti-hyaluronidase concentration of the serum of the normal male between the ages of 31 and 60 years remains constant at 28.2% ± 1.220 ($P.E._m$). However, the titre of the serum from the female between these same ages rises to 38.0% ± 1.926.

Accepting a 5% level of significance, it may be seen that the difference between the 2 titres is statistically significant for males and females above the age of 31. Since the majority of females studied, of the age 31 or over had entered or passed the menopause, it would seem that this endocrinological change is associated with an elevation in the anti-hyaluronidase titre of the serum.

The failure to recognize this difference in the titre between the sexes could easily lead to uncertainty in the interpretation of the anti-hyaluronidase level in serum from pathological conditions.

Conclusions. 1. The anti-hyaluronidase titre of the normal human serum has been determined by the means of the viscosimetric method.

2. The anti-hyaluronidase titre is the same for both sexes between the ages of 21 and 30 years (32.0%). Between the ages of 31 and 60 years the titre in the serum from the normal female (38.0%), is significantly higher than that of the male (28.2%).

The bull testicular hyaluronidase was furnished through the kindness of Dr. Irwin C. Winters, Director of Research, G. D. Searle and Co.

Received November 28, 1950. P.S.E.B.M., 1951, v76.

Serum Anti-Hyaluronidase in Human Leukemia and Lymphosarcoma. Effects of Cortisone and ACTH.* (18448)

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The present studies deal with the serum anti-hyaluronidase in human leukemia and lymphosarcoma, and with the changes in the levels following treatment with cortisone, ACTH and other forms of therapy. Follow-

* Aided by a grant from the Charles R. Blakely Fund of the National Research Council.

ing the work of Haas(1) on anti-hyaluronidase in infection, other workers studied the anti-hyaluronidase in malignant disease. Fulton *et al.*(2), found an increased titre in malignancy as compared to the normal. However, he did not separate his normal groups by sexes, nor did he differentiate the type of malignant disease studies. Hakanson and Glick(3), and Kirilik *et al.*(4), found that the anti-hyaluronidase levels were normal in patients with benign tumors, high in patients with cancer and highest of all in those whose cancers had metastasized. Most workers in this field have attempted to associate the spreading factor with the invasiveness of malignant tissue(5). The statement has been made recently that, "there is general agreement to support an association of spreading factors with carcinomas than there is with sarcomas"(6). However, Hakanson, and Kiriluk(3,4), both reported several lymphosarcomas which had the highest titre of all their cases.

There are no studies specifically on the anti-hyaluronidase titres in the blood of patients with leukemia or lymphosarcoma or the effects of cortisone or ACTH on the titre of these diseases. Our data indicate that very high anti-hyaluronidase titres are to be found in both these diseases, and that changes occur following treatment.

It has been shown(7), that the elevated anti-hyaluronidase titre of the serum in rheumatic fever is depressed upon the administration of cortisone or ACTH, and that this decrease parallels the clinical improvement. Dorfman(7), felt that this reflected changes in the tissue hyaluronic acid mechanism. The present data indicate that the serum anti-hyaluronidase parallels the state of the leukemia or lymphosarcoma just as it does in rheumatic fever. In acute or chronic lymphatic leukemia, a decrease in the titre occurs following the administration of cortisone or ACTH. However, in chronic myelocytic leukemia, cortisone causes an exacerbation of the disease process, and with it there occurs a marked rise in the anti-hyaluronidase.

TABLE I. Serum Anti-Hyaluronidase in Leukemia and Lymphosarcoma Untreated Cases.

Group	Sex	No. cases	Mean, %		Range, %
Normals	M	35	28.2		
	F	37	38.0		
Chr. lymph. leukemia	M	7	53.6	55.6	45.0-66.6
	F	4	58.7		48.5-76.5
Chr. myel. leukemia	M	5	49.8	49.7	43.7-53.0
	F	1	49.2		—
Acute myel. leukemia	M	1	52.4		—
Acute lymph. leukemia	M	1	47.6		—
	F	1	61.4		—
Lympho-sarcoma	M	2	71.0		57.5-84.5
	F	1	34.7		—
Hodgkin's disease	M	1	30.6	70.3	60.8-87.9
	F	3	70.1		

Methods. The viscosity method based on Haas(1), was used in preference to the turbidimetric or mucin clot methods since it was felt that viscosity more closely approximates the physiological function of hyaluronic acid.

Results and discussion. The results are summarized in Tables I, and II. It will be noted (Table I) that the leukemias and lymphosarcomas all uniformly show an elevate anti-hyaluronidase titre. The mean titre for 27 cases was 55.0%. This figure is similar to that of Kiriluk *et al.*(4) who found a mean of 57% for 3 cases of lymphoblastoma. It is of interest that the values for the lymphoid diseases are higher than for the myelocytic, and the value for the Hodgkin's-lymphosarcoma group is considerably higher than either form of leukemia (2 cases of the latter with low titres were found in patients whose disease was quiescent). Hence, the titre tends to be highest where the tumor cells invade or metastasize to the blood stream *least*, as in

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TABLE II. Effect of Therapy on the Anti-Hyaluronidase Titre.

Disease	Patient	Sex	Before therapy, %	After therapy, %	Treatment
Chronic lympho-cytic leukemia	L.	M	—	13.1	Urethane, NaPAB
	D.	M	75.7	40.5	ACTH 1.85 g
	R.	F	48.5	20.7	Urethane
	F.	F	69.0	30.8	ACTH
Acute lympho-cytic leukemia	E.	M	47.6	44.8	Amethopterin
	C.	F	61.4	36.4	Cortisone 1.55 g Nit. must. 30 mg
Chronic myelo-cytic leukemia	M.	M	53.0	17.6	X-ray, urethane
	M.F.	M	43.7	19.5	Urethane, nit. must.
	R.	M	49.8	60.0	Cortisone 1 g
	H.H.	F	—	13.8	Urethane
	E.C.	F	—	16.3	Fowler's sol.
	H.	F	49.2	71.1	Cortisone 1.2 g
Lymphosarcoma	N.	M	57.5	53.4	Cortisone 3.45 g
	E.F.	M	84.5	48.5	Cortisone 1.6 g
	B.	F	—	30.2	Urethane
	C.G.	F	34.7	53.2	Cortisone 2.95 g
Hodgkin's disease	A.	F	—	26.6	Testosterone, estrogens
	S.	F	72.5	56.6	Cortisone 1.1 g
	C.R.	F	61.7	39.4	Cortisone 1.5 g
	C.R.	F	62.0	37.0	Nit. must. 30 mg, X-ray

the latter disease. In addition, no correlation could be found between the anti-hyaluronidase titre and the bone marrow picture. Also, no marked differences were noted in the bone marrow when the titre was reduced by therapy. Our data, therefore, fail to indicate any relationship between the serum anti-hyaluronidase and the invasiveness of the tumor cells for the blood stream and bone marrow. The theory that the anti-hyaluronidase titre is a measure of invasiveness must assume, therefore, that Hodgkin's disease is invasive in the same sense as carcinoma. In 8 cases of carcinoma as well as the present studies, the impression is gained that the anti-hyaluronidase may be related more reasonably to the quantity of malignant tissue. In the leukemias and lymphosarcomas the quantity of tissue is great, and undoubtedly greater than in the majority of carcinomas by reason of their widespread distribution in many organs.

Following therapy, the anti-hyaluronidase titre dropped in conjunction with clinical improvement (Table II). Exceptionally low titres were found following the administration of urethane, either alone or in combination with other forms of therapy. This is of interest since urethane has a specific action upon nuclear metabolism. In general, it appears that a drop in the titre to normal or below normal was evidence of an arrest in the disease process.

Of particular interest is the very marked rise in anti-hyaluronidase attendant upon the administration of cortisone to 2 patients with chronic myelocytic leukemia. The titres rose to levels among the highest found in this study. This is indicative of the stimulating effect of cortisone upon the leukemic myelocytic cell.

Conclusions. (1) The anti-hyaluronidase titre of the blood of patients with lymphosarcoma, Hodgkin's Disease, and leukemia has been determined by means of the viscosimetric method. (2) The anti-hyaluronidase titre is elevated in all 3 conditions and varies with the clinical condition of the patient. (3) Cortisone causes an exacerbation of the disease in myelocytic leukemia with a corresponding increase in the anti-hyaluronidase titre. (4) In lymphocytic leukemia and Hodgkin's disease, cortisone, ACTH and other forms of therapy cause a decrease in the anti-hyaluronidase titre corresponding with clinical improvement. (5) There is no apparent relationship between the degree of invasion of the blood stream and the bone mar-

row picture and the changes in anti-hyaluronidase titre. (6) The height of the anti-hyaluronidase titre appears to be related to the quantity of tumor tissue rather than to its invasiveness.

Received November 28, 1950. P.S.E.B.M., 1951, v76.

Heinz Body Phenomenon in Monkey Erythrocytes, a Quantitative Method. (18449)

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Webster(1) has published an excellent review on the Heinz Body Phenomenon in Erythrocytes. In this he states that "further work on the relationship between Heinz body occurrence and the action of the spleen. . . . appears to be necessary in order to extend our knowledge of the role played by the spleen in hemolytic anemias produced by chemical agents." It has been observed that the amount of quinine hydrochloride necessary to produce the acute death of the monkey was less in animals with a severe anemia resulting from malaria than it was in monkeys with an equally severe anemia produced by phenylhydrazine(2).

Several methods have been devised for the demonstration and estimation of Heinz bodies. Cruz(3) and others(4-6) have made use of the turbidity which occurs in the blood to estimate quantitatively the amount of Heinz bodies present. Counts may be done either on fixed stained smears or on supravital preparations(7,8). Fertman and Doan(9) say, "The inclusions could be seen in unstained living blood films as refractile yellow tinted bodies. In dark field examination they appeared highly refractile, globular and irregular, similar in general appearance and distribution to the inclusions seen in bright field." Figge(10) thinks that Heinz bodies can be readily demonstrated in plain dried blood films examined with a high power objective; he found that they disappeared when examined under oil, balsam or other mounting media. Utilizing the fact that Heinz bodies are not hemolysed in distilled water, we have demonstrated that the number of Heinz bodies in the blood of the monkey may be determined by diluting the blood with water in a standard red cell pipette and counting the number of Heinz bodies on a haemocytometer. Observations were made in both the normal and splenectomized monkey.

Methods and materials. Ten *M. mulatta* monkeys, weighing from 2.6 to 4.0 kg, were used. Blood for the erythrocyte counts, smears and Heinz bodies was obtained from the marginal vein of the ears. Smears were stained with a combination of Wright's and Giemsa's stains. The number of Heinz bodies free in these smears was counted per 500 erythrocytes. Red cell counts were made using standard technics and Heinz body counts were carried out by a similar technic. The blood for the latter was drawn to the 0.5 mark on a red cell diluting pipette and the diluting fluid was drawn to the 101 mark, thus giving the count as the number of Heinz bodies in M per cmm. Distilled water and a solution of phloxine dye were used as the

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2. Rigdon, R. H., and Ruskin, Arthur, *J. Lab. and Clin. Med.*, 1949, v34, 1109.

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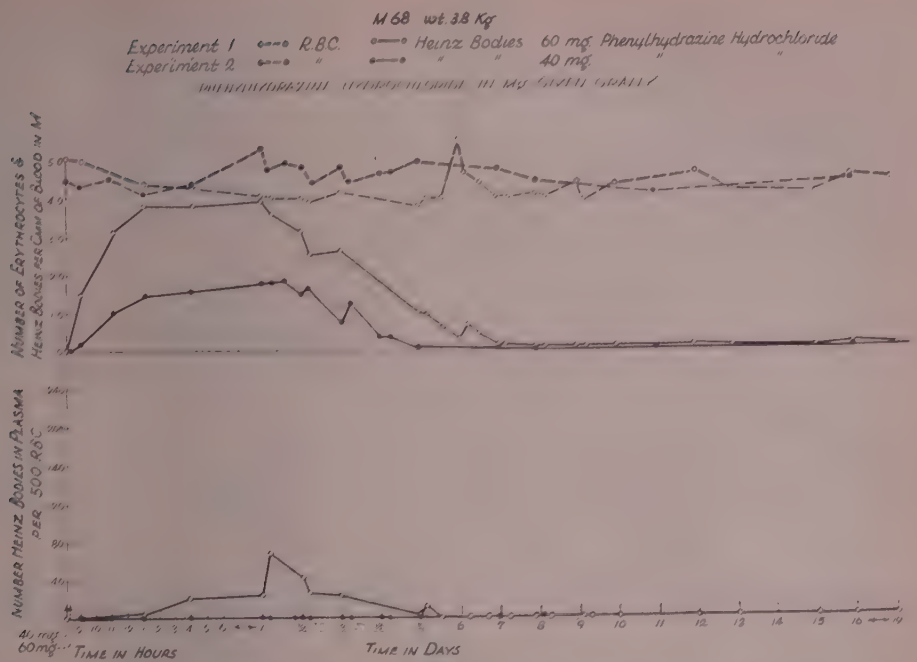

FIG. 1.

In Exp. 1, 60 mg of phenylhydrazine were given. The total erythrocyte count decreased approximately one million cells, however, the number gradually increased to approximately a normal level by the 20th day. The number of Heinz bodies as demonstrated by diluting the blood in distilled water rapidly increased during the first 24 hours of the experiment and then progressively decreased until the 8th day. The number of Heinz bodies free in the plasma was less than in the distilled water preparation. In Exp. 2, a smaller amount (40 mg) of phenylhydrazine was given. A corresponding decrease is reflected in the number of erythrocytes and Heinz bodies. In fact, there were too few to count per 500 erythrocytes during the time of the 2nd experiment. The time in hours shows that the experiment was begun between 8 and 9 A.M.

diluent. The phloxine solution was made from a stock solution in which 250 mg of phloxine powder were added to 500 ml of distilled water. A fresh solution was prepared using 5 ml of the stock solution in 200 ml of distilled water for each experiment. This dye was used to stain the Heinz bodies lightly. The blood in the pipette was shaken to assure mixing of the cells, then it was immediately placed upon a haemocytometer in the same manner as that used for an erythrocyte count. The Heinz bodies were allowed to settle on the counting chamber for 10 to 15 minutes before they were counted. The phenylhydrazine hydrochloride solution given to the monkeys was prepared by dissolving one gram of the drug in 50 ml of 95% ethyl alcohol. It was necessary to apply a little heat to dissolve the crystals. Although the preparation became deep yellow in color after a few days at room temperature, it was satisfactory for use. The drug was given orally through a small catheter. The amount given at one time varied from 20 to 150 mg. One to 3 such doses were given during an experiment. In some of the animals given phenylhydrazine hydrochloride, the number of Heinz bodies in the blood was followed for a month, at which time the spleen was aseptically removed. The monkeys again were given phenylhydrazine and the number of Heinz bodies was counted.

Heinz bodies in normal monkeys given phenylhydrazine. Seven normal monkeys, weighing from 3.2 to 4.0 kg, were given phenylhydrazine hydrochloride in amounts varying from 40 to 150 mg. Heinz bodies were demonstrated in the blood within an

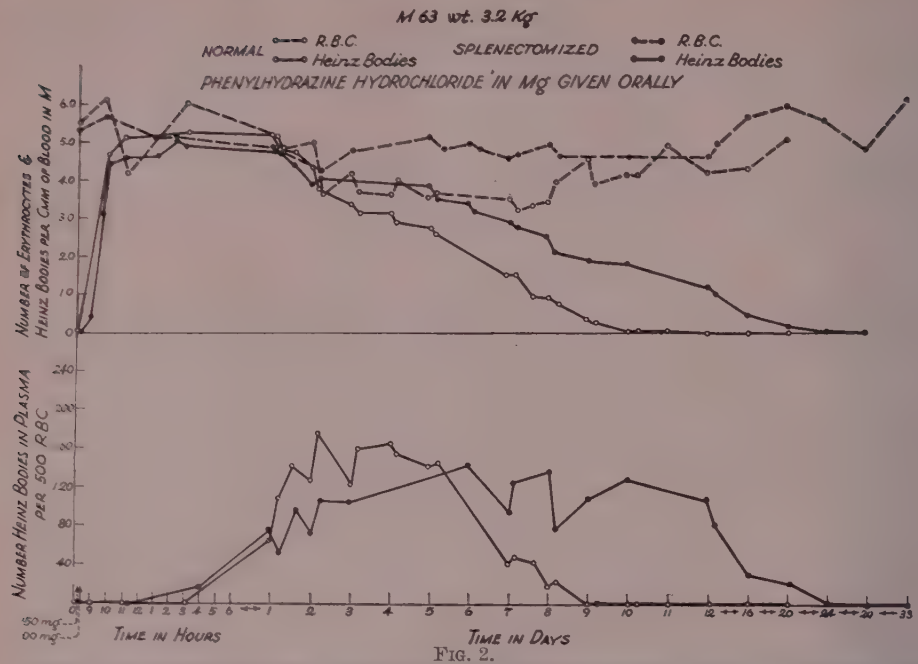

FIG. 2.

The number of Heinz bodies persists much longer in the circulating blood of splenectomized monkeys than in normal animals. The sequence of changes in the number of erythrocytes and Heinz bodies is the same as described in Fig. 1.

hour whenever the dose was 40 mg or more. The animals frequently became cyanotic after larger doses of the drug and their blood was chocolate brown in color. This color persisted for 2 or 3 days. The Heinz bodies seen within the first hour were very small and difficult to count. As time progressed, the Heinz bodies increased in size for approximately 36 hours.

Monkey 68 (Fig. 1, Exp. 1) was given 60 mg of phenylhydrazine hydrochloride. One hour later there were 1.51 million bodies per cm in the peripheral blood. Five hours later the Heinz body count was 3.89 million and the erythrocyte count was 4.30 million. Twenty-four hours later the Heinz body count was 3.94 million and erythrocyte count was 4.07 million. The number of bodies gradually decreased until the 19th experimental day, at which time none was demonstrated in the peripheral blood. The number of Heinz bodies free in the plasma during the time of this experiment is shown in Fig. 1. Seven days after Heinz bodies were last observed in Monkey 68, this animal was given 40 mg of phenylhydrazine hydrochloride (Fig. 1, Exp. 2). One hour later only 0.19 million Heinz bodies were present. The count reached its maximum of 1.86 million 32 hours later. The erythrocyte count at this time was 4.85 million. The number of Heinz bodies gradually decreased and the number of erythrocytes remained constant until the 7th experimental day, at which ime there were no Heinz bodies present. Although an occasional Heinz body could be seen within an erythrocyte in a blood smear, the number of bodies free in the plasma was too few to count per 500 red cells at any time during this 2nd experiment.

Monkeys 62 and 63 weighed 3.3 and 3.2 kg. They were given 150 mg of phenylhydrazine hydrochloride orally. The observations on Monkey 63 are shown in Fig. 2. Two hours after the phenylhydrazine was given, the Heinz body counts were 4.92 million and 4.66 million in these 2 animals. At this time their red cell counts were 5.29

million and 4.07 million, respectively. After 24 hours, Monkey 62 had 5.49 million Heinz bodies and 5.30 million erythrocytes. The number of erythrocytes and Heinz bodies in both of these monkeys gradually decreased until the 5th experimental day, at which time Monkey 62 had 3.19 million red cells and 2.47 million Heinz bodies, while Monkey 63 had 3.57 million erythrocytes and 2.70 million Heinz bodies. The number of Heinz bodies progressively decreased until the 12th experimental day when none was demonstrated. The number of erythrocytes in the peripheral blood returned to normal by the 30th experimental day.

Heinz bodies in splenectomized monkeys given phenylhydrazine. Splenectomies were performed on 5 monkeys, 4 of which had been given phenylhydrazine hydrochloride previously. They were given phenylhydrazine for a second time and the number of Heinz bodies was observed. Monkey 63 (Fig. 2) was given 100 mg of phenylhydrazine hydrochloride after splenectomy. One hour later the Heinz body count was 3.16 million and 4 hours later the count was 4.63 million. On the 10th experimental day the number of Heinz bodies had decreased to 1.89 million and the erythrocyte count was 4.63 million. The number of red cells had returned to normal by the 21st experimental day; however, Heinz bodies persisted free within the peripheral blood until the 27th day. The Heinz bodies free in the plasma reached their maximum number on the 6th experimental day. Ten days after the Heinz bodies were last seen in the peripheral blood, Monkey 63 was given 20 mg of phenylhydrazine hydrochloride. Three hours later there were only 0.27 million Heinz bodies in the peripheral blood. They reached their maximum number, 0.45 M, on the 14th experimental day. The number of erythrocytes was not affected by this small amount of phenylhydrazine. Heinz bodies persisted in a few red cells for 84 days. Monkey 62 was treated the same as Monkey 63. The clinical course and the hematological changes were essentially the same in these 2 animals.

Monkeys 46 and 52 were given phenylhydrazine hydrochloride in small doses and the number of Heinz bodies free in the plasma was observed. Monkey 52 was given 50 mg of the drug each day for 3 days. Heinz bodies remained in the plasma of this animal until the 37th experimental day. Monkey 46 was given 100 mg of phenylhydrazine and 2 days later another 50 mg. Visible changes in the erythrocytes were present on the 3rd experimental day. Heinz bodies remained in the peripheral blood for 37 days.

Monkey 67, weight 2.6 kg, was given 80 mg of phenylhydrazine hydrochloride. One hour later the Heinz body count was 2.93 million (Fig. 3, Exp. 1). The count reached its maximum 24 hours later, at which time there were 4.41 million Heinz bodies and the erythrocyte count was 4.39 million. The number of Heinz bodies and erythrocytes gradually decreased until the 7th day when the red cell count was 1.67 million and the Heinz body count was 0.49 million. The Heinz bodies were not demonstrable after the 20th experimental day and the number of red cells had returned to normal by the 27th day. The number of Heinz bodies in the plasma per 500 erythrocytes during the time of this experiment is shown in Fig. 3. Eight days after completion of experiment 1 a 2nd dose of 80 mg of phenylhydrazine hydrochloride was given. The results are shown in Experiment 2 (Fig. 3). They are essentially the same as observed in the first experiment.

On the 6th experimental day Monkey 67 was very sick and unable to eat. He died on the 19th experimental day with extensive tuberculosis. No Heinz bodies were demonstrated on the day of death.

Discussion. The monkey has been used infrequently for the study of Heinz bodies(7), but it is obvious from these experiments that it is a very satisfactory host for such observations. Although there may be a wide variation in the response of different monkeys to the same quantity of the drug, the data in Fig. 3 indicate that this experiment may be repeated in the same animal with essentially the same results.

The changes produced within the red cell by phenylhydrazine are present within 30 minutes after a large dose is given. At this early time there is no change observed within the

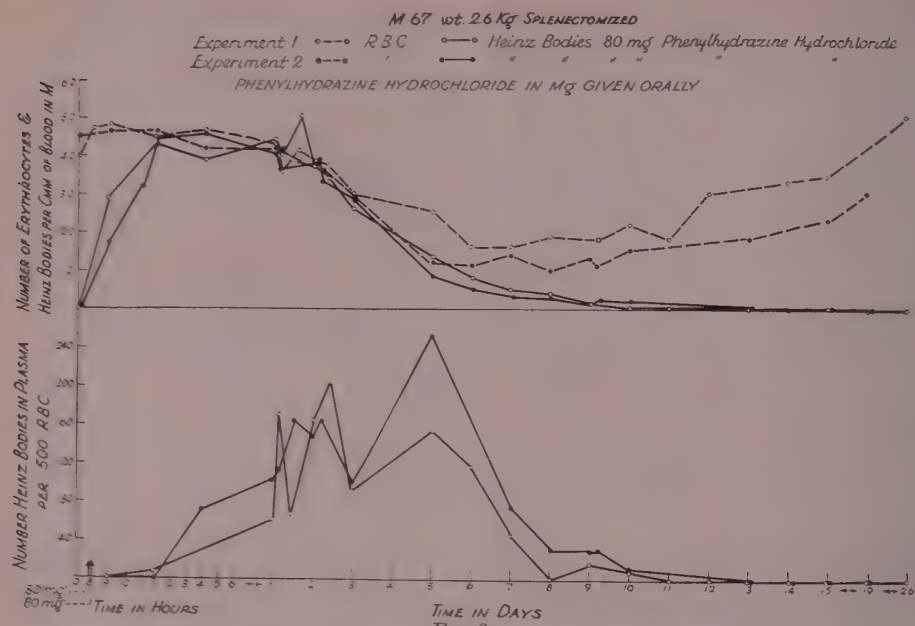

FIG. 3.

The experiment was repeated in this monkey. The results are essentially the same.

erythrocytes in routine blood smears stained with a combination of Wright's and Giemsa's stains. However, if at this time the red cells are put into distilled water, complete hemolysis does not occur. Subsequently, Heinz bodies become obvious within the erythrocytes and later these bodies become extracellular in blood smears. The change in the erythrocytes after a small dose of phenylhydrazine may be insufficient to result in free Heinz bodies in the blood smear (Fig. 1, Exp. 2). The number of Heinz bodies free in the plasma is proportional to the amount of the drug given. It should be noted, also, that the maximum number of Heinz bodies appears in the plasma following the time that the greatest number is present as shown by the direct counting method (Fig. 2 and 3). It is very difficult to count the Heinz bodies in blood smears when large amounts of the drug have been given since the cells and Heinz bodies are not distributed uniformly in the smear. Erythrocytes injured by phenylhydrazine hydrochloride are rapidly removed from the circulating blood of a normal monkey, while injured cells and Heinz bodies in splenectomized monkeys are removed at a much slower rate. Heinz bodies usually circulate in the peripheral blood of normal monkeys from 7 to 16 days and in splenectomized monkeys from 19 to 84 days. Cruz(3), using pyrodine, found that Heinz bodies remained in the peripheral blood of rabbits from 8 to 9 days and in dogs from 9 to 18 days. Webster and associates(7), using stibene, observed that these bodies persisted in the blood of guinea pigs for 11 days, in rats for 33 and in mice for 55 days.

The question arises of whether all erythrocytes injured by phenylhydrazine hydrochloride are removed from the peripheral blood. The resistance of some erythrocytes to the hemolytic action of distilled water would suggest that some of the cells, although affected, may continue to circulate for long periods. It is possible, also, that the change in the red cell is reversible when only a minimal change has been produced.

The method used in this study to determine the number of erythrocytes affected by phenylhydrazine hydrochloride is both simple and rapid. The ratio of Heinz bodies to the total erythrocyte count reflects the amount of phenylhydrazine hydrochloride absorbed.

In monkeys given a large amount of the drug, the number of cells affected is approximately the same as the total number of red cells. With smaller doses, the effect is proportionally less. Webster(1) comments on the fact that supravital preparations have "the advantage that the smaller Heinz bodies can be seen and thus the beginning stages of the phenomenon can be detected and followed." It should be remembered that a red cell affected by phenylhydrazine to such an extent that a Heinz body forms is also most likely to be affected to such a degree that either the cell or a portion of it will persist in distilled water and thus be counted in the total number of Heinz bodies. This technic could be used in routine clinical hematologic examinations and, if proven satisfactory, would be of value when drug or industrial poisonings are suspected.

Summary. Heinz bodies have been demonstrated in the blood of monkeys following the oral administration of phenylhydrazine hydrochloride. These bodies disappear from the circulating blood in normal monkeys more rapidly than they do from the blood of splenectomized animals. A quantitative method for determining the number of Heinz bodies has been described. This method is based upon the observation that Heinz bodies are not hemolysed in distilled water. A comparative study is made of the number of Heinz bodies free in the plasma in blood smears stained with a combination of Wright's and Giemsa's stains with those remaining after treatment with distilled water. The latter technic is both simple and rapid.

Received December 1, 1950. P.S.E.B.M., 1951, v76.

Adenosine Triphosphate from Barium-Acid Insoluble Residues. (18450)

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The purpose of this paper is to demonstrate that the customarily discarded barium-acid insoluble residues obtained in the classical methods for the extraction of adenosine triphosphate may contain considerable amounts of a Ba ATP which differs markedly in solubility from ordinary ATP and to outline a procedure for its isolation. We first became interested in these residues following a relatively unsuccessful attempt to prepare ATP from whelk muscle by the method of Needham(1). The yield from the barium-acid soluble fraction of this material was disappointingly low while the acid insoluble residue was many times larger than the corresponding fraction obtainable from mammalian muscle. The procedure reported here, originally worked out for dealing with the whelk residue, has also been applied to the much smaller residues from rabbit skeletal muscle and beef heart with qualitatively similar results.

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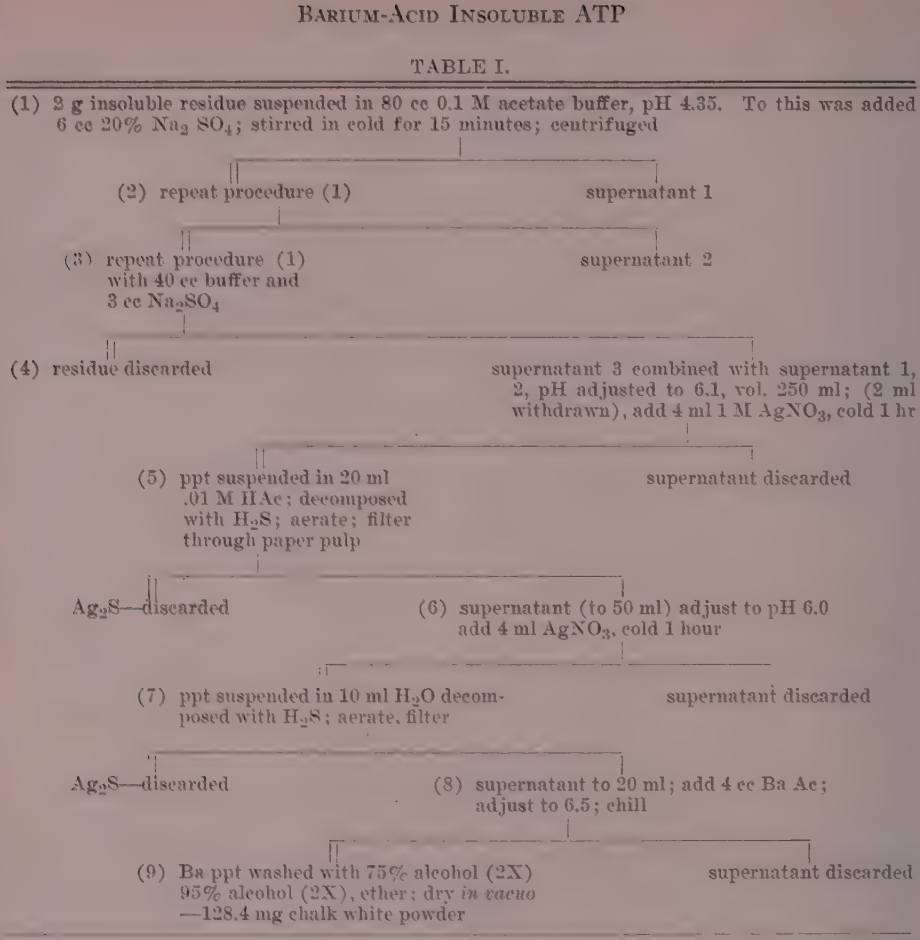
Methods and Results. From approximately a bushel of whelks (Busycon sp?), obtained from a local fish market, 4.082 kg of foot muscle were removed. The muscle was homogenized in Waring Blendors in cold 10% trichloracetic acid, filtered through cheese cloth, and reextracted with an equal volume of cold 5% T.C.A. The filtrates were combined and centrifuged in a Sharples centrifuge. The residue was discarded and the supernatant fluid adjusted to pH 8.2 with concentrated NaOH. A precipitate formed which was centrifuged out and discarded. The cloudy supernatant was treated with 1 volume of cold ethyl alcohol; the precipitate which came down was removed by centrifugation and to the supernatant solution (12.6 liters) was added another 6.3 liters of alcohol; no additional precipitate was formed. The clear supernatant fluid (approximately 18.0 liters) was treated with 300 cc of 25% barium acetate, and placed in the cold room over night. The next morning the barium pre-

TABLE I.



cipitate was collected, and suspended in H_2O; after centrifugation, the precipitate remaining was once more suspended in H_2O. The final precipitate obtained corresponds to the barium insoluble, alcohol insoluble fraction from which adenosine triphosphate is generally isolated.

This precipitate was homogenized and washed in 4 successive 300 cc aliquots of 0.2 N HNO_3. When rabbit muscle is used, most of the barium precipitate goes into solution, and a small residue remains which is discarded. In this case, however, the precipitate remaining was considerable; this was washed successively with barium acetate, alcohol, and ether and dried *in vacuo;* 6.3 g of a dry white powder were obtained. The material which had gone into solution in the nitric acid was treated with Lohmann's reagent and eventually precipitated as the barium insoluble salt; it corresponded to 359 mg of dibarium adenosine triphosphate, the molar ratio of total to labile phosphorus to adenine was 3.00:2.00:1.05.

Because of the low yield of adenosine triphosphate in the procedure described above, the nitric acid insoluble residue was reexamined. When small quantities of this material were suspended in H_2O, and the pH lowered to 1.0 little or none appeared to go into solution; after centrifugation the supernatant solution contained only traces of adenine as measured spectrophotometrically. The procedure outlined in Table I was therefore devised in an attempt to isolate additional material. In principle the method depends on bringing the nucleotides into solution by exchanging sodium for barium in suspensions

containing an excess of sodium sulfate, and then precipitating them as the silver salts. This method was first used as one of the purification steps in the isolation of adenosine triphosphate from plant tissues(2) where similar difficulties in purification were encountered.

By this procedure from 6.3 g of the insoluble residue 404 mg of a chalk white barium salt could be isolated. Analysis of this barium salt yielded the following molar ratio: total phosphorus : labile phosphorus : adenine:pentose, 3.02:1.86:1.07:1.00; purity based on pentose, 90% (calculated as Ba_2 ATP. $4H_2O$); inorganic P—none. Adenine was identified spectrophotometrically from the sharp absorption maximum at 2600 Å. In the orcinol pentose reaction(3) 74.0% of the color was developed in 7 minutes, as compared with 74% for vertebrate ATP. No free adenylic acid could be detected spectrophotometrically by the method of Kalckar using a muscle deaminase(4). The compound was indistinguishable in its behavior from ordinary vertebrate ATP in its ability to donate phosphorus to glucose in the yeast hexokinase system(5). (Table II).

This compound, however, was not readily soluble in water, even at pH 1 and had to be brought into solution by sodium exchange. When chromatographed on paper with a developer composed of 0.1 M malonate buffer at pH 6 and isoamyl alcohol, it appeared to be identical with ordinary ATP although it was evident that a very small amount of adenylic acid was present as a contaminant. But when the malonate buffer was replaced with a 0.1 M solution of barium acetate buffered at pH 4.7 with acetic acid, a marked difference in behavior was noted. With the latter developer, adenylic acid and ordinary ATP migrate at essentially the same rate and form sharply defined spots, while the compound prepared from whelk muscle leaves a long streak on the paper extending all the way from the starting point to the same level as that attained by the other compounds. This is taken to indicate that even in this relatively purified form, the solubility of the barium salt of the whelk compound differs from that of the usual mammalian preparation.

TABLE II.

Ability of Whelk and Vertebrate ATP to Donate Phosphorus to Glucose in the Yeast Hexokinase System, as Measured Spectrophotometrically. Decrease in density at 265 λ at the end of time in minutes.

		3	7	11	15	18
Sample						
A	Whelk ATP	.024	.058	.087	.105	.125
B	Vertebrate ATP	.024	.058	.084	.108	.122
C	Whelk ATP					+.005

Samples A and B each contained ATP, 0.5 ml 0.1 M malonate buffer pH 5.9, 0.1 ml $MgCl_2$ (10 mg/ml), 0.02 ml myokinase (containing 4 μg protein N), 0.1 ml 0.5 M glucose, 0.02 ml yeast hexokinase (containing 7 μg protein N), 0.03 ml adenylic acid deaminase (containing 15 μg protein N), and water to 5.0 ml. Sample C contained whelk ATP, malonate buffer, adenylic acid deaminase and water.

The same procedure was used on a nitric acid insoluble residue obtained from rabbit muscle and beef heart. From 159 mg of the rabbit residue was isolated 31.4 mg of a white barium salt which was indistinguishable from ordinary rabbit ATP. The molar ratio of total phosphorus : labile phosphorus : adenine : pentose was 3.00 : 2.00 : 1.06 : 1.00; purity based on phosphorus 83%. As in the case of the whelk, the compound contained no free adenylic acid and transferred its phosphorus to glucose in the yeast hexokinase system. By the same procedure, from 2.14 g of nitric acid insoluble residue from beef heart, was isolated 25.3 mg of a yellowish barium salt. The molar ratio of total phosphorus : labile phosphorus : adenine was 2.04 : 1.02 : 1.00, suggesting an ADP, rather than an ATP. That this compound was indeed an ADP is indicated by the observation that it is not deaminated directly by muscle deaminase, but is deaminated after first being treated with myokinase according to the method of Kalckar.

2. Albaum, H. G., Ogur, M., and Hirshfeld, A., *Arch. Biochem.*, 1950, v27, 130.

3. Albaum, H. G., and Umbreit, W. W., *J. Biol. Chem.*, 1947, v167, 369.

4. Kalckar, H. M., *J. Biol. Chem.*, 1947, v167, 461.

5. Albaum, H. G., and Lipshitz, R., *Arch. Biochem.*, 1950, v27, 102.

Whether these nucleotides are associated with some inert material from which they are removed with difficulty or whether they represent some polymerized form of these compounds, or both, we are not prepared to say at this time.

Summary. A procedure has been described for the isolation of adenosine triphosphate from barium-nitric acid insoluble residues prepared from whelk foot muscle and rabbit striated muscle. The ATP so isolated is indistinguishable physiologically from ATP prepared according to the standard procedure of Needham but is only slightly soluble in strong acid. It is present in the smooth muscle of the whelk in much larger amounts than in striated rabbit muscle. Treatment of a similar residue from beef heart yielded a compound which chemically and physiologically resembled ADP.

Received December 2, 1950. P.S.E.B.M., 1951, v76.

Effect of Intravenous Digitoxin on Fluid Distribution in Hospitalized Males without Cardiovascular Disease.* (18451)

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(Introduced by G. T. Harrell)

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Although the actions of digitalis on the heart are well known, its effect on the distribution of body fluids in normal individuals have not been reported. Because the cardiac glycosides are similar in chemical structure to other cyclopentenophenanthrene derivatives which are known to affect salt and water metabolism—for example, the sex hormones and the adrenal cortical hormones—they might be expected to produce some alteration in the distribution of fluids in the body.

The present study was conducted in order to determine the effect of intravenous digitoxin on the plasma and extracellular fluid spaces in normal individuals.

Material and Methods. Subjects. Serial studies were performed on 5 male patients who had been hospitalized for non-cardiovascular disorders and who had no obvious disturbances of salt and water metabolism. These individuals were ambulatory and were on a house diet. No attempt was made to control their intake of salt and water. *Material.* Commercial crystalline digitoxin, prepared from Digitaline nativelle and dissolved in a 40% solution of alcohol, was used in all cases. The solution contained 0.2 mg of digitoxin per ml of fluid. *Methods.* The methods which were used to determine the *hematocrit*, the *plasma volume* and the *thiocyanate space* have been described previously (1). In preliminary experiments it was demonstrated that the thiocyanate ion apparently reached a state of equilibrium in the extracellular fluid space 3 hours after injection(2); the 3-hour values, therefore, have been used for calculations and comparisons. Since the total volume of blood withdrawn for each complete study did not exceed 40 ml, the blood loss would not have affected the circulating blood volume appreciably. The *serum protein concentration* was determined by the Kingsley biuret method(3). This value and the plasma volume were used in calculating the *total circulating protein* content.

Plan of the experiment. A baseline deter-

* This work was supported in part by the U. S. Atomic Energy Commission through a contract with the Bowman Gray School of Medicine, and in part by a grant-in-aid from the American Heart Association.

† Research Fellow of the American Heart Association.

1. Aikawa, J. K., *Am. J. Physiol.*, 1950, v162, 695.
2. Aikawa, J. K., submitted for publication.
3. Kingsley, G. R., *J. Lab. and Clin. Med.*, 1942, v27, 840.

mination of all the quantitative measurements described above was made on each individual at least a day prior to the administration of digitoxin, and the subject was weighed at this time. After completion of the baseline studies, a total of 1.2 mg of digitoxin was administered intravenously to each patient. This amount was divided into 2 equal doses of 0.6 mg each, given one hour apart. Five hours after the first injection of digitoxin, venous blood was withdrawn and the solution containing T-1824 (Evans Blue) and sodium thiocyanate was injected intravenously. The plasma and total blood volumes were measured on samples drawn 10 minutes later. The thiocyanate space was calculated from a sample drawn 3 hours later (8 hours after the initial injection of digitoxin). The total serum protein concentration was determined on samples drawn 5, 7, and 8 hours after the first dose of glycoside; and the hematocrit, on the samples drawn at 5 and 8 hours. In order to determine whether the alterations in fluid distribution were reversible, the entire battery of tests was repeated 72 hours after the injection of digitoxin.

Method of statistical analysis. Each patient served as his own control. In order to test the significance of the results, the differences between the baseline value and the values observed at the specified time intervals after the administration of digitoxin were determined for each individual. The mean difference and the standard error were then calculated; from these values, "t" was obtained.

Results. (Table I). A progressive decrease in the mean serum protein concentration during the first 8 hours was found. The decrease at 8 hours (0.75 g per 100 cc) was statistically significant ($t = 8.4$). The mean values for the total circulating protein content before the administration of digitoxin, and 5 and 72 hours after its administration were 215, 235, and 200 g, respectively. The increase of 20 g at 5 hours was significant ($t = 4.95$, $P = < 0.01$).

A mean increase of 381 ml in the plasma volume and of 348 ml in the total blood volume (each roughly 10% of the baseline value) was found at 5 hours; the values at

TABLE I. Comparison of the Baseline and 5′ Plasma Volumes.

	Baseline	5′	d
Re	3272	3841	+569
Le	3492	3966	+474
Ca	3217	3841	+624
Br	2683	3004	+321
Be	2683	2596	— 87
		Sum of d	+1901
		Mean diff.	—380.2

$$S\,d^2 \quad 1,048,420$$

$$\frac{(Sd^2)}{n} \quad -722,760$$

$$325,660$$

$$Sd^2 - \frac{(Sd^2}{n} \quad 325,660$$

$$\frac{}{n(n-1)} \quad \frac{}{20} = 16,280.$$

$$\text{Square root} = S_d = 128.0.$$

$$t = \frac{380.2}{128.0} = 2.98.$$

72 hours were lower than the baseline values in 4 of the 5 subjects. The mean increase in plasma volume at 5 hours was at least suggestive ($t = 2.98$).

The decrease in the mean hematocrit value was not significant at either time interval. The decrease in thiocyanate space at 72 hours, although of considerable magnitude, was not significant. No change was observed at 5 hours. No significant alterations in body weight were noted during the period of observation, although there was a slight decrease at 72 hours.

Comment. The results of the present study suggest that changes occur in fluid distribution when 1.2 mg of digitoxin is given intravenously to individuals without cardiovascular disorders. A significant decrease in the concentration of serum proteins and an increase in the mean total circulating protein content, associated with a suggestive increase in the mean plasma volume and a decrease in the mean hematocrit, indicate that there is either (1) a primary shift of water and a secondary mobilization of labile protein into the vascular compartment or (2) a primary shift of protein containing fluid into the vascular compartment. The maximum dilution

of the blood proteins appears to have occurred at 8 hours. Unfortunately, the experiment was so designed that the plasma volume was not measured at that time.

It is known that in congestive heart failure the onset of diuresis is preceded by a fall in the specific gravity of the plasma, presumably due to an increased hydration of the blood plasma(4). The resultant increase in blood volume is thought to be responsible for the diuresis. A similar mechanism may have been in operation in the present study; this would explain the decrease in the thiocyanate space and in the body weight observed at 72 hours.

The changes which were observed in the fluid content of the vascular compartment may have been due to a primary decrease in cardiac output, with a consequent decrease in capillary pressure. However, a decrease in the venomotor tone, an increase in the interstitial fluid pressure, or both, may also cause the changes noted. Whether the results were due entirely to the glycoside's effect on the heart or whether there was also an extracardiac effect is not known. A more detailed study of the problem is now in progress.

Summary. The intravenous administration of 1.2 mg of digitoxin to 5 male subjects with no cardiovascular disorder was found to cause a significant decrease in the serum protein concentration and an increase in the total circulating protein content within 8 hours. The results were interpreted as indicating an increase in the water and protein content of the vascular compartment.

4. Stewart, H. J., *J. Clin. Invest.*, 1941, v20, 1.

Received December 4, 1950. P.S.E.B.M., 1951, v76.

Xanthine Oxidases in Different Species.* (18452)

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It has been assumed tacitly that the xanthine oxidase activities demonstrated in different organs from various species(1) were due to the same enzyme, and that this enzyme was similar or identical with the better-known milk xanthine oxidase. A previous study(2) showed that the enzyme occurring in rat liver was different from milk xanthine oxidase inasmuch as only the former could be inhibited by antabuse.† This antabuse inhibition was overcome by the addition of methylene blue to the aerobic system, or by heating the liver homogenate at 56° for 30 minutes. The latter treatment destroyed some of the original aerobic activity of the enzyme, but its dehydrogenating ability was not affected. This evidence was interpreted(2) as showing that xanthine oxidase occurred in rat liver as a complex of two distinct enzyme activities (dehydrogenase and oxidase) closely associated with some other unidentified constituent that conferred antabuse sensitivity upon the complex.

This report describes the application of the same techniques to other selected tissues in order to determine whether "xanthine oxidase activity" is uniformly due to the same kind of enzyme. Ignoring minor differences for purposes of generalization, the xanthine oxidase of mammalian tissues was similar to the rat liver enzyme. The xanthine oxidase of bird tissues, however, was fundamentally a dehydrogenase, since it was nearly devoid of autooxidizable characteristics. Evidence is

* This work was supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

1. Morgan, E. J., *Biochem. J.*, 1926, v20, 1282.

† Antabuse, tetraethylthiuram disulfide, was supplied by Ayerst, McKenna and Harrison, Ltd.

2. Richert, D. A., Vanderlinde, R., and Westerfeld, W. W., *J. Biol. Chem.*, 1950, v186, 261.

TABLE I. Effect of Antabuse, Methylene Blue, and Heating at 56° on Xanthine Oxidase Activities of Tissues from Different Species.

		Xanthine oxidase activities in cmm O_2/20 min./flask						
		Original tissue		+ antabuse (0.85 mg/cc)		Homogenate heated at 56° for 30 min.		
Tissue studied	No. of exp., avg	−	+ M.B.		+ M.B.	−	+ M.B.	+ antabuse
Rat liver*	6	34	60	12	60	15	55	19
" lung	3	8	10	4	9	13	10	11
" spleen	3	12	14	7	16	22	17	21
" kidney	3	2	6	1	7	3	6	2
" intestine†	3	9	24	0	18	11	30	0
	2	36	53	4	41	33	66	3
Mouse liver	3	40	59	8	59	22	59	23
Cat liver	5	12	31	3	30	12	31	7
Guinea pig liver	6	13	25	0	25	12	23	4
Dog liver	5	1	10	2	10	3	10	2
Rabbit liver	3	0	6	0	6	0	6	0
Pig "	2	11	17	1	17	11	14	8
Cow "	3	7	12	1	12	7	12	2
Frog "	2	4	4	0	4	0	3	0
Pigeon kidney‡	2	1	26	0	35	0	40	0
Chicken liver§	6	6	74	5	102	3	110	4
Turkey liver	3	0	64	4	67	1	77	1

* Published previously(2) but included for comparison.

† First group of 3 exp. was determined by tipping in xanthine substrate after 40-min. incubation. In next group of 2 exp. hypoxanthine substrate was added after 10 min. Results are not directly comparable since different rats were used.

‡ Values reported here may be lower than usually encountered, since about twice this activity was found in other incomplete exp.

§ Hypoxanthine substrate was added after 40-min. incubation.

not yet available which would allow a characterization of the "mammalian xanthine oxidase" as a complex of 2 separate enzymes (oxidase and dehydrogenase) so firmly bound together that they cannot be separated by the usual techniques, or whether this enzyme exhibits two activities because it contains 2 prosthetic groups within the same molecule. A study of purified chicken liver xanthine dehydrogenase is now in progress in order to determine the characteristics of the prosthetic group of this enzyme.

Methods. The xanthine oxidase activity of each tissue was determined manometrically by the procedure of Axelrod and Elvehjem (3,4). A weighed sample was homogenized with 2½ volumes of the phosphate buffer to give a concentrated homogenate. Aliquots were further diluted with buffer or buffer plus antabuse and rehomogenized to give a 1 + 5 dilution of the original tissue. The antabuse concentration was 1 mg per cc of homogenate or 0.85 mg per cc of fluid in the Warburg flask. Another aliquot of the concentrated homogenate was heated in a 56° water bath for 30 minutes, cooled, and then treated like the unheated sample. When necessary, tissues from several animals were pooled to make the concentrated homogenate. All Warburg flasks used in determining the xanthine oxidase activity contained 1.7 cc of homogenate (283 mg fresh tissue) in a total fluid volume of 2.0 cc. The effect of methylene blue was determined by making 0.15 cc of 0.0113 M methylene blue a part of the total 2 cc volume. The substrate, 0.15 cc of 0.05 M xanthine, was tipped into one of a pair of flasks after a preliminary 40-minute incubation period that partially exhausted the endogenous respiration. The activities have been recorded in net oxygen consumption per 20 minutes per flask, and are directly comparable.

Results. The results are shown in Table I.

3. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, v140, 725.

4. Richert, D. A., Edwards, S., and Westerfeld, W. W., *J. Biol. Chem.*, 1949, v181, 255.

From 2 to 6 complete experiments on each tissue were averaged to give the recorded values. In addition to the general conclusions recorded in the summary, the following points may be noted. Dog liver was previously reported(1) to be free of this enzyme, but its presence could readily be demonstrated by the addition of methylene blue to the aerobic test system. The pigeon was the only species in which the liver was found to be completely free of xanthine oxidase; no activity was detected in any of 6 experiments with xanthine or p-hydroxybenzaldehyde as the substrate in the presence or absence of methylene blue.

The xanthine oxidase of mouse liver was unchanged by a total body x-ray irradiation of 600 r. Control mice had liver xanthine oxidases in the presence and absence of methylene blue of 36 and 20 cmm O_2/20 min. respectively, while the mice analyzed 3 to 6 days after irradiation‡ had corresponding values of 42 and 18.

Human livers obtained at autopsy after accidental or other deaths had little xanthine oxidase. In the usual determination, values of 0 to 5 cmm O_2/20 min. were obtained, and these were increased 2 fold by the addition of methylene blue.

Summary. A study of the xanthine oxidases in selected tissue homogenates from different species showed that the enzymes present in bird tissues was a dehydrogenase rather than an oxidase, since the aerobic activity of the enzyme was negligible in comparison with its activity in the presence of methylene blue. The enzyme present in mammalian tissues was an oxidase whose aerobic activity could generally be increased from 1.5 to 2.5 times by the addition of methylene blue to the aerobic test system. In all cases the aerobic activity of the xanthine oxidase was inhibited from 40 to 100% by antabuse, and this inhibition was completely overcome by the addition of methylene blue. Heating the tissue homogenate at 56° for 30 minutes gave decreased aerobic xanthine oxidase activities with rat and mouse liver but no change with most mammalian tissues; the antabuse inhibition was eliminated to varying degrees with different tissues. The dehydrogenase enzyme or the dehydrogenase portion of the mammalian xanthine oxidase complex was unaffected by antabuse or by the heating procedure, since the activity in the presence of methylene blue remained constant irrespective of what happened to the aerobic activity.

Received December 6, 1950. P.S.E.B.M., 1951, v76.

‡ We are indebted to Dr. L. B. Clark of Union College for the irradiated and control mice used in these studies.

Reactions of Albino Rats to Injections of Dextran. (18453)

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Dextran, a long-chain polysaccharide, in 6% concentration in physiological saline solution, has been used as a plasma substitute in the treatment of hemorrhagic and burn shock (1-4). In experiments with this substance in the albino rat, consistent reactions have been observed.

Reactions in 29 albino rats, 150-250 g, have been observed in our laboratory when 0.5 ml 6% Dextran per 100 g body weight was injected into the tail vein. All rats showed reactions, lasting for a period of 2 or

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1. Gronwall, A., and Ingelman, B., *Nature*, 1945, v155, 45.

2. Bohmansson, G., Rosenquist, H., Thorsen, G., and Wilander, O., *Acta Chir. Scand.*, 1946, v194, 149.

3. Lundy, J., *et. al.*, *Proc. Staff Meet. Mayo Clinic*, 1947, v22, 357.

4. Turner, F. P., *et al.*, *Surg. Gynec. and Obst.*, 1949, v88, 661.

FIG. 1.
Showing swelling of snout in rat after injection of Dextran.

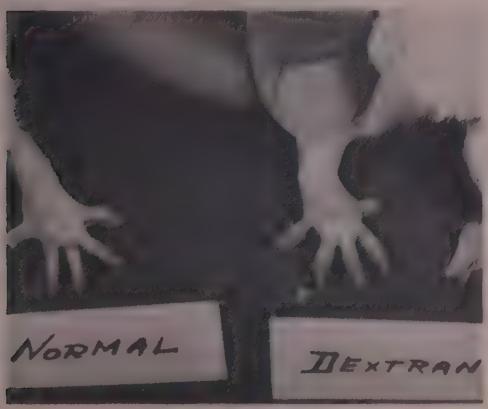

FIG. 2.
Showing swelling of paw in rat after injection of Dextran.

3 hours. Stupor, dyspnea and plethora of ears and feet was observed after 5 minutes, followed frequently by scratching. In 15 minutes swelling of snout and paws was evident. (Fig. 1,2.) The plethora, dyspnea and stupor subsided in 30 to 60 minutes and the swelling was diminished or absent after 2 hours. These reactions of the albino rat to Dextran have been observed in the laboratories of Dr. Walter Bloom(5) of Emory University and Dr. W. D. A. Maycock of the Lister Institute, London.

In another experiment, 27 rats were injected intraperitoneally with 6% Dextran, 1 ml per 100 g body weight. The reactions were prominent in every case and consisted of the same elements. The only difference noted was a 30-minute delay in the onset of reactions with those animals injected intraperitoneally.

To test the relation of rat reaction to human reaction, 3 pairs of rats were injected intraperitoneally, each pair receiving one of the following lots of Dextran: (1) from a bottle which had produced a reaction in a human; (2) from a lot which had not produced any reaction in human trials, and (3) from a lot which had not had a human trial. The reactions of these rats showed no significant variation.

To relate the molecular weight of Dextran to the reaction, 6% solutions of the following mean molecular weight fractions were employed: (1) 170,000; (2) 130,000; (3) 80,000; (4) 40,000; and (5) 25,000. One ml per 100 g body weight of each fraction was injected into 2 rats intraperitoneally. Equal amounts of 6% stock Dextran and isotonic saline were injected into control rats.

Table I shows that the reaction occurs between 15 and 30 minutes after injection with 25,000 molecular weight fraction, and between 30 and 45 minutes with stock Dextran and fractions 40,000 to 80,000 molecular weight. Rats receiving fraction 130,000 molecular weight showed the first reaction after 45 minutes, and those receiving 170,000 molecular weight fraction had no reaction until after one hour. In addition to slower onset, the reactions were not as severe when the intraperitoneal injection was with fractions of higher molecular weight. When these fractions were administered intravenously, following the same plan (Table II), the animals reacted to all fractions in the first 5 minutes. The reactions were as marked with the larger molecular weight fractions as with the smaller.

When Dextran was administered intravenously on subsequent days, the degree of reaction diminished (Table III). The diminution of the redness following the subsequent injections was striking. The most persistent sign of reaction on subsequent injections was the swelling of loose tissue on

5. Morrison, J. L., Bloom, W. L., and Richardson, A. P., *J. Pharm. and Exp. Therap., Proceedings*, Jan. 1951, v101, 27.

TABLE I. Dextran Reactions to Various Molecular Fractions Injected Intraperitoneally.

Fraction	II	III	IV	V	VI	Stock sol. dextran 170,000 to 40,000	Isotonic saline
Mean molecular wt	170,000	130,000	80,000	40,000	25,000		
Time after inj., min.							
15	0	0	0	0	0	0	0
30	0	0	0	0	+	+	0
45	0	0	++	++	++	+	0
60	0	+	++++	++++	++++	+++	0
75	+	++	++++	++++	++	++++	0
90	+++	+++	+++	++	+	++	0
105	+	++	++	++	+	+	0
120	+	++	++	++	+	+	0
135	−	−	−	−	−	−	0

+ to ++++ indicates severity of reaction (plethora, swelling, dyspnea, scratching).

TABLE II. Dextran Reactions to Various Molecular Fractions Injected Intravenously.

Fraction	II	III	IV	V	VI	Stock sol. dextran 170,000 to 40,000	Isotonic saline
Mean molecular wt	170,000	130,000	80,000	40,000	25,000		
Time after inj., min.							
3	+++	++++	+++	+++	+++	++	0
15	++++	+++	+++	++++	++	++	0
30	+++	+++	+++	++	++	+++	0
45	+++	++	++	++	++	++	0
60	++	++	++	++	++	++	0
75	++	++	++	++	++	++	0
90	++	++	++	++	++	++	0
105	++	++	++	++	+++	++	0
120	++	++	++	++	+++	++	0
135	++	+	+	++	+++	++	0

+ to ++++ indicates severity of reaction (plethora, swelling, dyspnea, scratching).

TABLE III. Severity of Reactions to Intravenous Dextran Administered on Subsequent Days.

Day Inj.	1	2	3	4	5
1	+++	++	+	++	+
2	+++	++	++	+	+
3	+++	++	++	+	+
4	+++	++	+	+	+
5	++	+++	+	+	+

+ to ++++ indicates severity of reaction (plethora, swelling, dyspnea, scratching).

face and paws.

Summary and Conclusions. Albino rats give a demonstrable reaction of redness, swelling of loose tissue, pruritis and stupor, when injected with doses of Dextran comparable to recommended doses for humans. Three different lots of Dextran gave the same reactions. Fractions of varying mean molecular weight all gave prompt reactions when administered intravenously. The delay in reaction following intraperitoneal injection may be related to the time required for the Dextran to enter the general circulation in sufficient quantity. The larger molecules pass more slowly into the circulating blood. Dextran reactions in rats following injections on subsequent days are diminished.

Received December 8, 1950. P.S.E.B.M., 1951, v76.

Renal Sodium Handling under Prolonged Pentobarbital Anesthesia.* (18454)

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Certain advantages are offered by the convenient anesthetic agent, sodium pentobarbital, for investigation of renal problems where surgical intervention is required in acute experiments, including ease of administration and, generally, lack of effect on filtration rate and renal plasma flow(1). During the course of such investigations it was desired to know if pentobarbital significantly influenced the tubular mechanism for sodium reabsorption, a possibility suggested by the finding of an anti-diuretic-like action of the barbiturates(2). ADH is known to be natriuretic(3). The present report gives the results of observations on the sodium mechanism in dogs subjected to an anesthetic depth suitable for surgery maintained for approximately 5 hours.

Methods. Three trained female dogs weighing from 11.3 to 13.7 kg were used in these studies. In 3 experiments in each animal, following suitable control periods, 30 mg per kg of pentobarbital were administered intravenously. Additional small amounts were given to maintain uniform anesthesia as evidenced by respiratory rate and lid reflexes. Isotonic saline was given by stomach tube about one hour prior to the beginning of each experiment in an amount of 50 cc/kg of body weight. Simultaneously, 3 g of creatinine were injected subcutaneously. An intravenous priming dose of one-half gram of creatinine was given prior to the beginning of the clearance observations and again midway during the experiment. Each experiment was divided into 4 stages (with one exception of 3 stages), each consisting of usually 2 and sometimes 3 consecutive urine collection periods of about 15 minutes duration, with intervals of one to 1½ hours between stages. Urine was collected by indwelling catheter, and 2 rinses of distilled water followed by air insufflation terminated each period. Blood was drawn early and late in each stage from the jugular vein. During the experiment the dogs lay loosely

TABLE I. Renal Sodium Mechanism Under the Action of Sodium Pentobarbital Anesthesia.

Dog	Time, min.	P_{Na}, mEQ/L	C_{Cr}, cc/min.	Load, mEQ/min.	UV, mEQ/min.	Reabs., mEQ/min.	%† reabs.
A	C*	138.2	69.0	9.535	.055	9.480	99.4
	67	138.0	69.0	9.514	.236	9.278	97.5
	180	138.3	67.8	9.365	.178	9.187	98.2
	315	138.7	68.4	9.483	.140	9.343	98.1
B	C	141.7	48.2	6.810	.039	6.770	99.4
	73	141.6	48.2	6.832	.031	6.800	99.5
	185	142.0	57.8	8.175	.138	8.037	98.4
	291	141.4	52.2	7.376	.103	7.273	98.5
C	C	133.5	58.5	7.833	.027	7.806	99.7
	82	133.0	50.6	6.735	.030	6.705	99.5
	204	133.2	59.3	7.860	.069	7.791	99.2
	285	137.4	55.3	7.591	.027	7.564	99.7

* C = Control. Each datum in the table is avg of 3 exp. in each dog. Time is given from time of inj. to end of observation period.

† % of load reabsorbed averages 99.46 (S.D. ± 0.31) for control, and 98.75 (S.D. ± 0.78) for the periods during pentobarbital action. The standard error of the difference between means is 0.180, and P = <.01, a statistically significant difference.

* Supported by grant from the U. S. Public Health Service.

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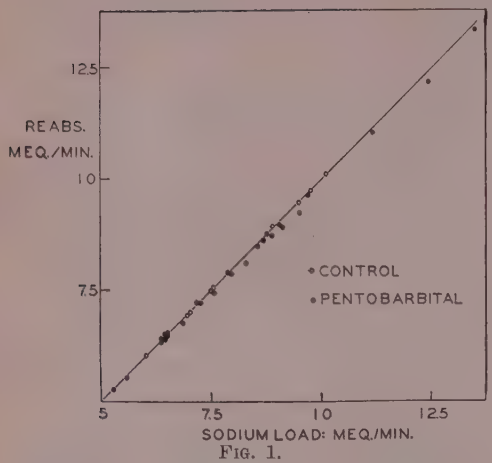

FIG. 1.

Showing the relationship of sodium reabsorption to load (filtration rate × plasma sodium conc.) in 9 exp. in 3 dogs. Duration of anesthesia was approximately 5 hr.

restrained on a padded dog board.

Creatinine clearance was used to measure glomerular filtration rate. Its concentration was measured in tungstate filtrates of the plasma and diluted urine by the alkaline picrate method. All analyses were in duplicate. Sodium was measured in diluted plasma and urine with an internal standard flame photometer. A correction factor of 0.95 was applied to the plasma sodium concentration for the Donnan effect.

Results. Emphasis is placed in Table I on the influence on tubular reabsorption of sodium as given in the last column in terms of the percent of the filtered load which is reabsorbed by the tubules before and during anesthesia. During the control periods an average of 99.46% (S.D. ± 0.31) is reabsorbed in all experiments. With pentobarbital, reabsorption averages 98.75% (S.D. ± 0.78) in all animals. Fig. 1 graphically illustrates the relation of reabsorption to load for each stage of observation. The solid line shows the trend of the control periods.

When the data on sodium excretion in Table I are examined critically it is seen that in every animal a perceptible, though variable, increase results during anesthesia. On the average, this represents an increase in excretion of 66 micro-equivalents over the control average of 41 micro-equivalents per minute. This increase in excretion results from the less than one percent decrease in reabsorption noted above. When this difference in reabsorption was analyzed statistically, it proved to be significant (P of $<.01$). However, in terms of physiological alterations generally observed during experimental procedures, *e.g.,* sodium loading(4) such small variations are considered unimportant.

Summary and conclusions. The influence of prolonged light surgical anesthesia with sodium pentobarbital on the renal tubular sodium reabsorptive ability was investigated in dogs initially given 50 cc per kg of isotonic saline by stomach tube. During time intervals averaging 5 hours, the tubules continued to reabsorb an average of 98.75% of the filtered load. The conclusion is reached that this anesthetic agent has a negligible effect on sodium reabsorption.

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Received December 11, 1950. P.S.E.B.M., 1951, v76.

Propagation of Pathogenic Fungi in the Yolk Sac of Embryonated Eggs.* (18455)

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Investigations now in progress in this laboratory show that the yolk sac of embryonated eggs provides superior nutritional and environmental conditions for the propagation of pathogenic fungi. That the chick embryo method should prove to be useful for the study of

* Aided by grants from the Eli Lilly Co.

fungi was first suggested by Goodpasture(1). The observations reported by Moore(2) on the infection of the chorio-allantois with several fungi represent the only significant contribution to this problem up to the present time. The preliminary results of studies now in progress will be presented in the present report.

Methods. The present studies began with an attempt to produce infection of the chorio-allantois with *Actinomyces bovis*. The extra embryonic membranes were found to be indifferently suited for this purpose. Chance inoculation of the yolk sac resulted in the abundant growth of *A. bovis*. This observation led to a more thorough investigation of the possibilities of this method of inoculation for the propagation of pathogenic fungi and the study of mycotic infections. Embryos of 7 to 9 days incubation proved to be most suitable for this purpose and were used throughout. The microorganisms were introduced into the yolk sac of living embryos through a small slit in the eggshell by means of a tuberculin syringe and 22 gauge needle. The initial inoculum in the case of most of the fungi studied consisted of a crude saline suspension of the microorganism cultured on media appropriate for it. Embryos were sacrificed at daily intervals following inoculation. The course of infection was observed by studying appropriately stained smears prepared from aspirated yolk or from small portions of yolk membrane. Subcultures to artificial media suited to each type of fungus were made at regular intervals. The entire yolk of selected embryos were fixed in Zenker's (10% acetic acid) solution from which suitably sized blocks were cut for embedding and microscopic section. As indicated below most of the fungi studied were propagated in series in the yolk sac through few to numerous transfers. Subinoculations for passage to fresh embryos were usually made on the 5th or 6th day following inoculation.

Results. A brief summary of the results obtained with each of the fungi investigated in this manner will be given as follows.

Actinomyces bovis. A saline suspension prepared from a blood agar shake culture† was used to initiate infection of the yolk sac of 8- to 9-day embryos. Within 48 to 72 hours numerous microorganisms typical of *A. bovis* were demonstrable in Gram stained smears of aspirated yolk. Transfers to fresh embryos were made on the 5th or 6th day following inoculation. The inoculum consisted of 0.1 ml of infected yolk emulsified by shaking in a flask with sterile glass beads. Thirty-two successive uninterrupted passages were maintained before the series was purposely terminated. Microscopic sections of the infected yolk sacs show numerous clumps of the microorganisms with typical "ray fungus" appearance. Control studies indicate that *A. bovis* is maintained in its typical morphological and cultural characteristics by passage in the embryonic yolk sac. Embryos survive yolk sac infection until hatching. Hatched chicks develop normally although *A. bovis* can be recovered from the absorbed yolk sac in 1-month-old chicks inoculated during incubation. Other such chicks kept until 6 to 8 months of age showed no evidence of infection and were free of *A. bovis*.

Nocardia asteroides. This microorganism grows very readily in the chorioallantois as well as in the yolk sac. In both of these sites *N. asteroides* proliferates so rapidly that the embryo is overwhelmed and dies within 3 to 4 days following inoculation. It apparently can be maintained indefinitely in serial passage in the membrane or within the yolk sac. The limited observations made indicate that much can be learned regarding the behavior of this group of microorganisms by a study of its effect on the chick embryo.

Nocardia intracellularis.‡ Unlike *N. asteroides* this microorganism thrives very poorly in the chorio-allantois but grows readily in the yolk sac. Within 48 to 72 hours following inoculation numerous microorganisms can be demonstrated in Gram or acid fast stained smears prepared from the yolk. It has been maintained through 5 successive transfers.

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† Obtained from Dr. Norman F. Conant.

‡ Obtained from Dr. Norman F. Conant.

Further detailed studies are required for an elucidation of the behavior of this interesting microorganism.

Sporotrichum schenkii. Infection of the yolk sac of 8-day-old embryos was initiated with a 10% saline suspension prepared from a subcutaneous nodule removed surgically from a patient with typical sporotrichosis. Within 72 hours after inoculation Gram stained smears show the yolk to contain both the cigar-shaped bodies and mycelial elements of the microorganism in abundance. Microscopic sections reveal that *S. schenkii* localizes in the immediate vicinity of the cells of the yolk membrane. It remains to be determined whether or not continued passage will result in the exclusive adaptation of the cigar-shaped bodies or of the mycelial form. Studies of its spread from the yolk to the embryo should prove to be rewarding.

Histoplasma capsulatum.§ Infection of the yolk sac of 7- to 9-day-old embryos was readily initiated with 0.1 ml of a slightly turbid saline suspension of the yeast form of this fungus. Within 48 to 72 hours and from then on until hatching heavily encapsulated forms are seen in abundance in smears stained by Wright's or Giemsa's method. Embryos survive to hatching without evidence of infection. The yeast form has been maintained by serial passage at 8 to 10 day intervals over a period of 6 months. Attempts to establish infection of the chorio-allantois with the yeast form obtained from blood agar slant cultures were consistently unsuccessful. Inoculation with yolk from the 10th passage produced grossly visible lesions in the membrane within 72 to 96 hours. Smears from the lesions show the encapsulated microorganism in abundance. Sections indicate a widespread intracellular invasion of the fibroblasts and monocytes of the mesoderm. Inoculation of the yolk sac with a suspension of the mycelial phase produces a rapid and complete transformation into the yeast phase within 48 to 72 hours.

Cryptococcus neoformans. Infection of the yolk sac was initiated with 0.2 to 0.3 ml of spinal fluid from a clinical case of the disease. This microorganism increased in numbers with marked rapidity within the yolk sac as demonstrated by examination of fresh mounts or India ink preparations. Exceedingly large capsules are elaborated. Invasion of the embryos occurs without apparently adversely affecting its development. Encapsulated yeast cells are readily demonstrated in the liver of chicks hatching under these circumstances.

Coccidioides immitis. Inoculation of the embryonic yolk sac with a suspension of the mycelial growth of a culture of *C. immitis* results in its rapid and almost complete reversal into the spherule phase. Detailed studies will be required to elucidate all of the steps which can readily be observed in this transformation. This microorganism is best observed by means of fresh mounts of the infected yolk. Studies are now in progress in an effort to adapt it to the yolk sac by continuous passage.

Discussion. These preliminary observations are presented with the purpose of indicating the great potentialities of the chick embryo method for the propagation of pathogenic fungi and for the study of the behavior of these microorganisms. As in the case of other pathogens, *e.g.*, the rickettsiae, the yolk sac, rather than other parts of the embryonated egg, has been found to be the site of choice for investigative work with pathogenic fungi.

The method has proven of considerable aid for the diagnostic isolation of some of these agents. By the addition of 50 units of penicillin and 50 mg of streptomycin per cc to exudates or suspensions of lesions obtained from 3 different patients, *Actinomyces bovis* was isolated and identified by yolk sac inoculation. *Sporotrichum Schenkii* has been thus far isolated and identified by this means in 2 separate instances. It should prove of considerable value in the isolation of *Histoplasma capsulatum* as well as other mycotic agents of disease. A new approach is also offered for the testing of the effectiveness of therapeutic and antibiotics by the yolk sac method. The present studies are being pursued further with especial emphasis on the early stages of the pathogenesis of these various mycotic

§ Obtained from Dr. Norman F. Conant.

agents of disease.

Summary. The yolk sac of the developing chick embryo has proved to be highly suitable for the propagation of the following pathogenic fungi: *Actinomyces bovis, Nocardia asteroides, Nocardia intracellularis, Sporotrichum Schenkii, Histoplasma capsulatum, Cryptococcus neoformans* and *Coccidioides immitis.* This method promises to be of great value for the experimental study of these mycotic infections. It is quite likely that many other of the pathogenic as well as non-pathogenic fungi may be studied with profit by infection of the yolk sac of chick embryos.

Received December 12, 1950. P.S.E.B.M., 1951, v76.

Effect of Cortisone on Passively Induced Skin Hypersensitivity in Man. (18456)

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Beneficial effects following the use of ACTH and cortisone have been described in status asthmaticus, hay fever, serum sickness, atopic dermatitis and gastrointestinal allergy (1-7). The tuberculin test has also been inhibited by these two agents(8,9) and cortisone has prevented anaphylactic shock in sensitized mice(10). Germuth and Ottinger (11) demonstrated that the concomitant administration of a sensitizing antigen and either ACTH or cortisone to normal rabbits prevented the development of hypersensitivity and suppressed antibody formation. The same animals, however, manifested the Arthus phenomenon if passively sensitized. Although these reports suggest that cortisone and ACTH have an effect on immune responses, certain manifestations of hypersensitivity have not been suppressed by the administration of these hormones. ACTH or cortisone, given prior to the injection of a shocking dose of tetanus antitoxin horse serum in sensitized guinea pigs, failed to alter the subsequent course of anaphylactic shock(8,12,13). The administration of cortisone to sensitized rabbits has also been ineffective in altering the active or passive Arthus phenomenon (8,14).

The present study was undertaken to determine the effect of cortisone on passively

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induced hypersensitivity in man. Under the conditions of this experiment, cortisone did not effect the immediate wheal and erythema type of skin hypersensitivity in passively sensitized human beings.

Materials and methods. The method employed for the demonstration of passive skin sensitization was essentially that described by Prausnitz and Küstner(15). Two patients under treatment with cortisone served as donors. Serum of one patient suffering from severe serum sickness was obtained after 4 days of treatment with cortisone in a total dose of 600 mg. The other serum was drawn from a patient with an obscure type of diffuse vascular disease who had an incidental ragweed hypersensitivity manifested clinically by hay fever and a positive skin test to ragweed pollen extract. This serum was obtained after the patient had been treated for 13 days with a total dose of 1500 mg of cortisone. The recipients were volunteers selected from hospital patients who had no clinical history of serum sickness or hay fever and who were not sensitive by skin test to the materials employed in the experiment. Six volunteers who were not receiving cortisone served as controls. Fourteen volunteers were selected from patients under treatment with cortisone for a variety of illnesses in doses of at least 100 mg daily for from 3 to 37 days at the time the Prausnitz-Küstner test was performed. After passage through a Swinney filter, the donors' sera were diluted 1:5 and 1:10. These sera in dilutions of 1:100 produced positive Prausnitz-Küstner reactions inconsistently in the control group of volunteers. The experiments were therefore carried out with the lower serum dilutions. Dilution of all materials was performed with sterile isotonic saline. One tenth of a milliliter of the diluted sera was injected intradermally into each of 4 sites on the arm, thigh or back of the volunteers. The 4 passively sensitized sites were tested 24 to 48 hours later by the intradermal injection of approximately 0.025 ml of the test solutions as follows:

Site No. 1 was tested with the appropriate antigen, either tetanus antitoxin horse serum diluted 1:10, or ragweed pollen extract containing 500 total nitrogen units per ml. This site was prepared to determine whether a positive Prausnitz-Küstner reaction could be elicited and to serve as a control for the second site. Site No. 2 was tested with the appropriate antigen, to which cortisone had been added in a concentration of 2.5 mg per ml, in an attempt to increase the concentration of cortisone at the locus of antigen-antibody union. Local injection of higher concentrations of cortisone were found to be impractical for reasons discussed below. The cortisone was prepared from a suspension of cortisone acetate containing 25 mg per ml (Cortone, Merck). Site No. 3 was tested with the appropriate antigen to which was added, in a 1:10 dilution, the vehicle employed for suspending cortisone. This vehicle contained 1.5% benzyl alcohol, 0.4% polyoxyethylene sorbitan monooleate, 0.5% sodium carboxymethylcellulose, and 0.9% sodium chloride in distilled water. This test was done as a control in the event of inhibition of the reaction at site No. 2. Site No. 4 was tested with the suspension of cortisone alone in order to demonstrate that a wheal at site No. 2 represented a specific skin test for the antigen and not a skin reaction to cortisone. The cortisone was diluted in order to obviate a wheal and erythema reaction found to occur in a significant number of cases when the undiluted cortisone preparation was injected intradermally. This reaction was found to be due in part to the suspending agents and preservatives in the vehicle.

Skin tests with the above preparations were performed at distant unsensitized sites as controls. The skin reaction of a wheal and erythema was observed after 15 to 20 minutes and graded from 1 to 4+. Reactions showing wheals more than 2.0 cm in diameter were recorded as 4+, 1.5 to 2.0 cm as 3+, 1.0 to 1.5 cm as 2+, and distinct wheals less than 1.0 cm as 1+. In 4 volunteers the experiment was performed both before and during treatment with cortisone to determine whether a partial, if not complete, inhibition

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TABLE I. Passive Transfer of Skin-Sensitizing Antibodies from Patients Receiving Cortisone to Volunteers Also Receiving Cortisone.

Volunteer	Skin tests at passive transfer and distant control sites with: Horse serum diluted 1:10	Control	Horse serum diluted 1:10 and cortisone 2.5 mg/ml	Control	Ragweed extr. 500 N units/ml	Control	Ragweed extr. 500 N units/ml and cortisone 2.5 mg/ml	Control
E.*	++	0	+++	0	++	0	++	0
Y.	++++	0	++++	0	+++	0	+++	0
M.	++	0	+++	0	++	0	++	0
C.	+++	0	+++	0	++	0	++	0
A.	+++	±	+++	±				
A.Y.	++	±	++	±	+	0	+	0
M.C.	++	0	++	0	++	0	++	0
I.	+++	0	+++	0				
B.	++	0	++	0				
M.O.	++	0	++	0	++	0	+++	0
C.M.	++	±	++	0				
I.	++	±	++	±				
I.G.	+	0	+	±				
J.	++	±	++	0				

* Volunteer received 300 mg of cortisone per day.

of the Prausnitz-Küstner reaction by cortisone could be noted. It was also considered of interest to determine simultaneously the effect of cortisone on the tuberculin test. Three volunteers, who were found to have a positive Mantoux test with a 1:100 dilution of old tuberculin prior to treatment with cortisone, were retested while receiving cortisone. The tuberculin tests were read after 48 hours and the area of induration measured.

Results. Positive skin tests were consistently demonstrated in all of the 5 volunteers, not receiving cortisone, who were passively sensitized with serum from a patient receiving cortisone for 4 days in a total dose of 600 mg for the treatment of severe serum sickness. Similarly, 5 volunteers showed a positive reaction when skin tested after passive sensitization with the serum of a patient with ragweed hay fever receiving a total dose of 1500 mg of cortisone in 13 days. In none of these volunteers did the addition of cortisone locally, combined with the antigen employed in performing the skin test, reverse the reaction.

Positive skin test reactions to horse serum were demonstrated in all of the 14 volunteers receiving cortisone treatment who had been passively sensitized with the serum of the patient with serum sickness under treatment with cortisone. All 7 of the same group of volunteers who were passively sensitized with the serum of the patient with ragweed hay fever receiving cortisone treatment, also showed a positive skin test to ragweed pollen extract. In no instance did the local addition of cortisone reverse or partially inhibit the positive skin test. The results of these experiments are summarized in Table I.

In 4 cases the Prausnitz-Küstner reaction, elicited with both antigen-antibody systems, was compared before and during treatment of the volunteer with cortisone. No significant quantitative difference in the reactions was noted and therefore, partial inhibition of the reaction by treatment with cortisone was not demonstrated. The results of this experiment appear in Table II.

In one volunteer (E.), the dose of cortisone was raised from 100 mg to 300 mg per day. Passive sensitization was carried out on the third day of treatment and skin tests with the appropriate antigens were performed on the fourth day. Despite this relatively large dose of cortisone, no inhibition of the Prausnitz-Küstner reaction was noted when compared with the same reactions elicited before cortisone therapy was instituted.

TABLE II. Comparison of Prausnitz-Küstner Reaction Before and During Treatment of Volunteers with Cortisone.

Volunteer	Antigen preparation	Skin tests at sites passively sensitized with serum from patients with serum sickness and hay fever treated with cortisone: Before cortisone	During cortisone
C.	Horse serum diluted 1:10	+	+++
	Horse serum dil. 1:10 and cortisone 2.5 mg/ml	+	+++
	Ragweed extr. 500 N units/ml	++	++
	Ragweed extr. 500 N units and cortisone 2.5 mg/ml	++	++
M.	Horse serum diluted 1:10	+++	++
	Horse serum dil. 1:10 and cortisone 2.5 mg/ml	+++	+++
	Ragweed extr. 500 N units/ml	++	++
	Ragweed extr. 500 N units and cortisone 2.5 mg/ml	++	++
Y.	Horse serum diluted 1:10	+++	++++
	Horse serum dil. 1:10 and cortisone 2.5 mg/ml	+++	++++
	Ragweed extr. 500 N units/ml	+++	+++
	Ragweed extr. 500 N units and cortisone 2.5 mg/ml	+++	+++
E.*	Horse serum diluted 1:10	+++	++
	Horse serum dil. 1:10 and cortisone 2.5 mg/ml	+++	+++
	Ragweed extr. 500 N units/ml	++	++
	Ragweed extr. 500 N units and cortisone 2.5 mg/ml	++	++

* Volunteer received 300 mg of cortisone per day.

Three volunteers showed a moderately positive tuberculin reaction to a dilution of 1:100 of old tuberculin before treatment with cortisone. After receiving cortisone for three days in a total dose of at least 500 mg, only one of these volunteers showed a significant partial reversal of the tuberculin reaction. This was demonstrated simultaneously with the failure of the same subject to show any inhibition of the Prausnitz-Küstner reaction. This case therefore demonstrated a difference in the effect of cortisone on the two classical types of skin hypersensitivity.

Discussion. Under the conditions of the above experiments, cortisone does not interfere with the passive transfer of skin sensitizing antibodies, nor does it inhibit the wheal and erythema type of hypersensitivity resulting from the union of antigen and sensitizing antibody in the skin of human beings. These results are in agreement with reports of similar studies performed in experimental animals(8,11-14).

The experimental data do not preclude the possibility that larger doses of cortisone may inhibit or reverse the Prausnitz-Küstner reaction. However, the dosage employed was usually adequate to achieve a clinical response of the diseases for which the volunteers were treated. Beneficial effects with similar doses of cortisone have been reported in various allergic diseases usually associated with the immediate type of skin hypersensitivity (3,4). The fact that only one of 3 of our volunteers showed definite inhibition of the tuberculin test does not imply that the dose of cortisone was inadequate since one volunteer in whom such inhibition was not manifest was receiving 300 mg of cortisone per day while the test was performed. It has also been noted by other observers(9,14) that the reversal of the tuberculin reaction is not a consistent phenomenon in human beings treated with cortisone. However, in the volunteer in which reversal occurred, it was not associated with a similar reversal of the Prausnitz-Küstner which was simultaneously elicited.

The mechanism by which cortisone may favorably influence the clinical course of hypersensitive states is not known. The evidence presented favors the hypothesis that such improvement as may be noted during treatment with cortisone operates through mechanisms other than the inhibition of the union of antigen and sensitizing antibody(14). In this respect it is interesting to note that direct skin tests with the offending antigen were not suppressed in three cases of bronchial asthma who showed marked clinical im-

provement on ACTH(1). It is also of interest that some patients receiving ACTH have been observed to develop urticaria as a manifestation of hypersensitivity to the ACTH preparation itself(16).

Summary. (1) Positive Prausnitz-Küstner reactions were consistently demonstrated in volunteers passively sensitized with sera from 2 hypersensitive donors treated with cortisone. (2) Positive Prausnitz-Küstner reactions were similarly demonstrated in *cortisone treated* volunteers passively sensitized with sera of the cortisone treated donors. (3) There was no significant difference in the Prausnitz-Küstner reactions of those volunteers tested before and during treatment with cortisone. (4) It may be concluded that under the conditions of the experiments described, cortisone does not influence passively induced skin hypersensitivity in man.

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Received December 13, 1950. P.S.E.B.M., 1951, v76.

Diet of Mother and Vitamin B_{12} Content of Tissues of Infant Rats. (18457)

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The vit. B_{12} content of various organs and tissues of the rat was determined by a rat growth assay method and the kidney contained the largest amount(1). The vit. B_{12} content of the liver, spleen, heart and kidney of 28-day-old rats from mothers receiving the basal diet and the basal diet supplemented with vit. B_{12} concentrates from different sources is reported.

Experimental methods. The composition of the basal diet of the mothers is given in Table I. The females in Group 1 received the basal diet. Those in Group 2 received a supplement of 2.0 g of Merck's APF concentrate No. 3 per kilo of diet. Those in Group 3 received 1.0% of Lederle APF concentrate and those in Group 4 received 3.0% of liver "L." The mothers received the experimental diets from 28 days of age until they had an opportunity to produce at least 4 litters. The young in a few litters from each group were killed at 28 days and the tissues of 2 to 4 rats in each litter were combined into one sample. There were 1 to 3 samples of tissues per litter, depending upon the number of young in the litter. For example, if there were 10 young in a litter the tissues from 4, 3 and 3 rats respectively were combined to give 3 different samples. Vit. B_{12} was liberated from the tissues by the procedure described by Couch *et al.*(3) and

TABLE I. Composition of the Basal Diet.

Basal mixture	%
Cerelose	57.8
Casein	30
Woodpulp	3
Mineral mixture(2)	5
Wesson oil	4
Vit. supplement per 100 g basal mixture	
Vit. A	3000 I.U.*
Vit. D	425 I.U.
	mg
Menadione	2.5
Alpha tocopherol	2.5
Thiamine hydrochloride	1
Riboflavin	1
Pyridoxine hydrochloride	1
Ca pantothenate	4
Niacin	5
Choline chloride	100
Biotin	.02
Inositol	10
Para amino benzoic acid	50

* Vit. A and D were supplied by Mead Johnson's oleum percomorphum.

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TABLE II. Effect of Vit. B_{12} in Diet of Mother on Vit. B_{12} Content of Tissues of Young.

Group No.	Source of vit. B_{12}	No. of rats	No. of samples	Avg μg B_{12} per 100 g tissue			
				Liver	Spleen	Heart	Kidney
1	None	14	4	6.1 (4.1- 7.3)*	7.7 (7.0- 8.3)	1.7 (1.4-2.3)	11.3 (8.8-13.8)
2	Merck APF No. 3 2 g/kg (55 μg B_{12})	49	17	8.2 (4.0-13.9)	11.4 (1.7-19.5)	4.2 (1.5-7.0)	10.6 (6.2-17.9)
3	Lederle APF concentrate 10 g/kg (6 μg B_{12})	13	5	8.4 (5.2-10.9)	11.7 (10.1-15.5)	2.9 (2.2-3.9)	13.5 (10.5-19.3)
4	Liver "L" 30 g/kg (37 μg B_{12})	18	6	12.9 (7.5-16.8)	12.0 (10.1-14.6)	3.4 (1.4-5.5)	10.8 (7.3-15.1)

* Numbers in parentheses equal range.

TABLE III. Amount of Vit. B_{12} in Different Samples of Tissue from the Same Litter.

Litter No.	No. of tissues in sample	μg of B_{12} per 100 g tissue							
		Liver		Spleen		Heart		Kidney	
		Single	Avg	Single	Avg	Single	Avg	Single	Avg
1	2	4.0		1.7		5.4		11.2	
		6.2	5.1	13.8	7.8	4.5	5.0	7.2	9.2
2	4	13.9		14.2		3.6		17.9	
	3	12.2	13.1	14.8	14.5	7.0	5.3	10.2	14.1
3	3	4.9		9.5				8.8	
	3	7.4	6.2	10.7	10.1	2.3	2.3	9.1	9.0
4	4	4.1				1.5		12.4	
	4	5.9	5.0	8.3	8.3	2.3	1.9	10.1	11.3
5	4	16.8		12.0		5.5			
	3	15.7		10.1		3.0			
	3	16.5	16.3	14.6	12.2	3.5	4.0	15.1	15.1
6	3	10.9		15.5					
	3	9.3		11.9		2.2		19.3	
	2	10.0	10.1	10.1	12.5	3.9	3.1	10.5	14.9

was determined in the extract by the method described by Skeggs *et al.*(4). Thiomalic acid was added to the medium as a growth factor for *L. leichmannii* 4797 (ATCC) and as a reducing agent to protect vit. B_{12} during autoclaving. Crystalline vit. B_{12} was used as the standard and all the assays were autoclaved for only 5 minutes prior to inoculation. Acid production was used to measure growth response.

Results and discussion. The range and the average amounts of vit. B_{12} per g of tissue are given in Table II. The amount in the liver ranged from an average of 6.1 μg B_{12} per 100 g for those receiving the basal diet to 12.9 μg for those receiving liver "L." The livers from the groups receiving Merck's and Lederle APF concentrates contained an average of 8.2 and 8.4 μg B_{12} per 100 g respectively.

The spleens from the basal group contained an average of 7.6 μg of vit. B_{12} per 100 g and when the mother received a vit. B_{12} concentrate it was increased to a range of 11.4 to 12.0 μg B_{12} per 100 g. The hearts from the basal groups contained 1.7 μg of vit. B_{12} per 100 g and those from the groups receiving a source of vit. B_{12} ranged from 2.9 to 4.2 μg B_{12} per 100 g. The vit. B_{12} content of the kidney ranged from an average of 10.6 to 13.5 μg B_{12} per 100 g and the difference between groups was not important.

4. Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, v176, 1459.

In a comparison of the amount of vit. B_{12} in different tissues, the kidney contained more than the liver with the exception of the group receiving liver "L," which is in agreement with the work of Lewis, Register, and Elvehjem(1). When the diet contained liver "L" the liver contained slightly more than the kidney. The spleens contained approximately the same amount as the kidney with the exception of those in the basal groups. The spleens of this group contained slightly more than half that in the kidney. The amounts in the heart were relatively small compared with that in the other tissues.

The amounts of vit. B_{12} in different samples of tissue from the same litter are given in Table III. There it may be seen that the values were in the same range, with the exception of those for the spleen in litter 1, for all the samples within a litter.

Summary. The amount of vit. B_{12} in the liver, spleen and heart of 28-day-old rats was approximately doubled when a source of vit. B_{12} was added to the diet of the mothers. The diet of the mother had no important effect on the vit. B_{12} content of the kidney. In general the kidney and spleen contained slightly more vit. B_{12} than liver. The heart contained a relatively small amount of vit. B_{12} in comparison to other tissues.

The authors are indebted to Merck and Co. for vitamins and APF No. 3, to Lederle Laboratories for folic acid and APF concentrate, and to Wilson Laboratories for supplying liver "L."

Received December 18, 1950. P.S.E.B.M., 1951, v76.

Infra Red Spectroscopy of Tissues. Effect of Insulin Shock.* (18458)

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Infra spectra of organic compounds are with a few exceptions, highly characteristic and specific. For this reason study of infra red spectra of tissues has been considered as an adjunct method of tissue analysis(1). In our attempt at evaluating the potential use of infra red spectroscopy for tissue studies 2 characteristics of this physical method became apparent. 1. The locations of absorption bands of molecules are so extremely sensitive even to slight changes of molecular structure that it is necessary to resort to empirism and to draw conclusions about the structures by correlation between bands which occur regularly with recurring structural features throughout a series of material examined. 2. The infra red energy emitted by all commercial instruments is so small that loss of energy as may occur in examination of very small structures may make qualitative observations inaccurate and quantitative studies futile. The present paper is a report on our method for infra red spectroscopy of tissues, on qualitative spectroscopy of various tissues especially neural tissue, and on quantitative infra red spectroscopy in brain tissue of control rabbits and rabbits in insulin shock. For this study we have selected to investigate tissue masses rather than smaller units. The selection of brain areas, *viz*: diencephalon, hippocampus, cerebellum, and tectum was largely governed by the clinical manifestations of disordered neural functions displayed during insulin shock.

Preparation of tissue sections. The organs of the animals were rapidly frozen with dry ice immediately after the animal was killed, in almost all instances, by injection of air into the heart. For spectroscopy, frozen sections of 50 μ and, for some purposes, 25 μ thickness prepared by the technic of Adam-

* This work was aided by a research grant to the Diabetic Coma Project from the National Institute of Health, U. S. Public Health Service.

1. Blout, E. R., and Mellors, R. C., *Science,* 1949, v110, 137.

stone and Taylor(2) were placed on silver chloride plates and dried at low temperature. Strict adherence to this technic is necessary, as is the selection of homogenous sections. As a check on the exact location of areas studied spectroscopically and to rule out the possibility of complicating disease, histologic preparations were made from the block remaining after cutting the tissue for spectroscopy.

Method of qualitative infra red spectroscopy. For recording of the spectra with the Beckman 1R2, infra red spectrophotometer, the silver chloride plate was placed in a cell holder, the desired part of the section centered, and then covered with a specially designed mask so that no infra red energy would pass through any area of the tissue or part of the plate not covered by the section. Most of our tissue sections were obtained from an area of 8 x 5 mm. In some instances, spectra were recorded from larger, not infrequently, from smaller areas. Areas as small as 3 x 3 mm. can be studied without exceeding the proper signal to noise ratio of the instrument. Blanks were recorded from every silver chloride plate using masks with the same surface area and the same slit width of the instrument. Proper corrections were applied for the different amplifications.

Results of qualitative infra red spectroscopy of the tissues of rabbits. The results given here represent a selection of data obtained in a study of spectra from well over 500 tissue sections of control rabbits and rabbits in insulin shock. A large number of spectra were recorded from different parts of the brain (cerebellum, hippocampus, tectum, diencephalon, white matter, grey matter), a smaller number from liver, heart, medulla and cortex of the kidney, adrenal, thymus, and spleen, and in a few instances from optic nerve and lymph node. The recordings from 15-2.8 μ showed a region below 8.06 μ (1241 cm^{-1}) with several more or less strong bands which can be found in almost all tissues. These bands, with the exception of the band at 8.06 μ for which no definite assignment has yet been made, may be correlated with the known "protein polyamide" vibrations, *viz;* 3.04 μ (3209 cm^{-1}) N H stretching; 3.4 μ (2940 cm^{-1}) C H bending; 6 μ (1670 cm^{-1}) C O stretching, and the deformation vibration of N H at 6.44 μ (1553 cm^{-1}) and the deformation vibration of C H at 6.88 μ (1450 cm^{-1})(3). In the region of higher wave length (lower frequencies), the tissue spectra showed a fingerprint region which is so specific for individual organs that it was frequently possible to identify the tissue from the infra red spectra. Table I shows the location of bands from 11-8 μ in control rabbits and rabbits in insulin shock.

Several hundred sections of neural tissues examined by infra red spectroscopy showed without exception the same spectral pattern, *e.g.,* a weak band at 10.27-.30 μ and a strong band at 9.31-.35 μ. It is noteworthy that these bands could be seen at exactly the same locations in sections from different parts of the brain, sciatic nerve, and optic nerve of the rabbit, in the brain tissue sections from fresh human post mortem material, in the brain sections obtained from an anesthetized dog and that the strong band at 9.31-.35 μ was previously found by other investigators in the nerve of the frog(4). Great differences, however, existed in the strength of these bands. This will be discussed with quantitative determination of infra red spectra.

A relative small number, *e.g.,* a total of 76 visceral tissue sections were studied and only noteworthy findings can be discussed. More details are contained in Table I. A band which was very similar in strength to the weak band found in neural tissues was observed at somewhat similar location (10.32 $\pm$ .06 μ) in all visceral tissues except the liver of control animals. The visceral tissue spectra showed also one or more additional bands between 9.89 and 9.16 μ. The locations of these bands were distinctly different from those found in neural tissue (9.31-.35); they were somewhat similar in sections from thy-

2. Adamstone, F. B., and Taylor, A. B., *Stain Technology,* 1948, v23, 109.

3. Darmon, L. E., and Sutherland, G. B. B. M. Y., *Am. Chem. Soc.,* 1947, v69, 207.

4. Barer, R., Cole, A. R. H., and Thompson, H. W., *Nature,* 1949, v163, 198.

TABLE I. Location of Bands from 8-11 μ in Animal Tissue.

Type tissue	No. rabbits	Location and strength† of bands					
Brain, optic sciatic N.*	25	10.30-.272		9.35-.314			8.063
Liver							
Control	5	10.271	9.70-.644		9.23-.212	8.64-.632	8.073
Insulin shock	5	10.30-.282			9.23-.214	8.64-.611	8.063
Insulin no shock	2	10.35-.282			9.23-.224	8.602	8.05-.033
Insulin no shock	3	10.261	9.66-.624		9.22-.212	8.66-.632	8.08-.063
Insulin rapid shock	2	10.27-.261	9.68-.664		9.22-.212	8.642	8.063
Heart							
Control	3	10.30-.271		9.504	9.24-.222	8.561	8.07-.043
Insulin shock	4	10.27-.261		9.54-.524	9.25-.182	8.61-.561	8.08-.053
Insulin shock	1	10.281		9.501	9.204	8.561	8.063
Kidney-Medulla							
Control 4							
Insulin shock 3	7	10.311		9.47-.444			8.043
Insulin shock	6	10.33-.271			9.234		8.073
Kidney-Cortex							
Control 3							
Insulin shock 5	8	10.31-.281-2			9.30-.224 (Broad)		8.073
Adrenal							
Control	3	10.35-.301	9.822	9.60-.562	9.26-.173	8.52-.491	8.053
Insulin shock	7	10.38-.261	9.89-.842	9.651	9.27-.233	8.491	8.053
Repeated insulin shock	1	10.381	9.892	9.651	9.381	8.511	8.053
Thymus							
Control 3							
Insulin shock 5	8	10.36-.332			9.22-.164		8.063
Spleen							
Control 3							
Insulin shock 7	10	10.38-.302			9.22-.234		8.073
Lymph node							
Insulin shock	1	10.382			9.174		8.073

* Control and treated animals gave same results. † = 1 very weak. 2 weak. 3 medium. 4 strong.

mus, spleen and lymph node (9.23-.16), but clearly distinguishable in all the other tissues. A strong band at 9.64-.70 μ and a weak band at 9.21-.23 μ, for example was only seen in liver tissue. Some visceral tissue sections from animals which had received insulin showed a shift of the strong band toward lower wave length. This was especially apparent in the liver from 7 insulin animals in which the band at 9.64-.70 μ failed to appear and the band at 9.21-.23 had become very strong, the strongest band of the finger print region. Larger number of animals of different species must be studied in order to verify these findings.

Structural assignments of the tissue spectra. Blout suggested similarity of infra red spectra of nucleic acids and certain bands he found in a few tissue spectra. We have studied infra red spectra of ribose nucleic acid, desoxyribose nucleic acid, d-ribose, d-desoxyribose, nucleotides, purine- and pyrimidine derivatives and compared our data with those of Blout(5), Clark(6), and Randall *et al.*(7) and will report our findings in detail elsewhere. We feel that although about 100 different compounds related to nucleic acid metabolisms have been evaluated still more pure compounds and mixtures of pure compounds will have to be studied before any definite assignments of tissue spectra should be attempted. For the tentative assignment of the weak band found in neural tissue at 10.27-.30 μ and in visceral tissues at 10.32 $\pm$ .06 μ, the

5. Blout, E. R., and Fields, M. J., *Biol. Chem.*, 1949, v178, 335.

6. Clark, C. C., Infra red absorption and x-ray diffraction of nucleic acid derivatives, University Columbia 1950 P.H.D. Publication 1838.

7. Randall, H. M., *et al.*, Infra Red Determinations of Organic Structures, D. Van Nostrand, New York, 1949.

following findings in pure compounds may be significant: A strong band was found at 10.35 μ in ribonucleic acid, at 10.28-.30 μ in desoxyribonucleic acid (Bios), and at 10.25 μ in adenosine, weaker bands were found at 10.40 μ in xanthine, at 10.37 μ in hypoxanthine, at 10.26 μ in adenine sulfate, and at 10.25 μ in A. T. P. (di barium salt). Uric acid did not show any band at all at this wave length range. In the other part of the finger print region (9.89-9.16 μ) where most of the tissue spectra showed rather strong bands, differences between d-ribose and d-desoxyribose (Bios) were apparent. D-ribose showed a weak band at 9.19-.23 μ and a strong band at 9.63 μ; d-desoxyribose on the other hand showed a strong band at 9.22 μ and a weak band at 9.82-.85 μ. The spectra of liver tissue from control animals were very similar to those of d-ribose. When a shift of the strong band appeared in liver tissue from animals which received insulin that band had shifted to 9.22-.20 μ; that is almost exactly the location of the strongest band of d-desoxyribose. Evaluation of the strong bands of thymine, uracil, guanine hydrochloride, adenine sulfate, xanthine, hypoxanthine, and uric acid proved that none of those compounds possessed any band comparable with strong bands of the tissue spectra between 9.70 and 9.16 μ. Adenine sulfate showed a medium strong band at 9.78 μ and uric acid a medium strong band at 9.75 μ. It is, therefore, improbable that any of the strong bands found in the tissues at this wave length range, may have been caused by free purines or pyrimidines. Nucleosides, nucleotides, and nucleic acids, however, showed a large number of bands which may have to be considered for the assignment of the strong bands in the tissue spectra. The locations of strong bands of some of the pure compound are as follows: adenosine 9.63 μ, uridine 9.46 μ, cytosine sulfate 9.28 μ, yeast adenylic acid 9.75 μ, yeast guanylic acid 9.50 μ, uridylic acid 9.71 and 9.57 μ, cytidilic acid 9.25-.26 μ, ribonucleic acid 9.56 μ, and desoxyribonucleic acid 9.89 and 9.39 μ. In an attempt at obtaining further information about the structures which produce the infra red spectra in neural tissue

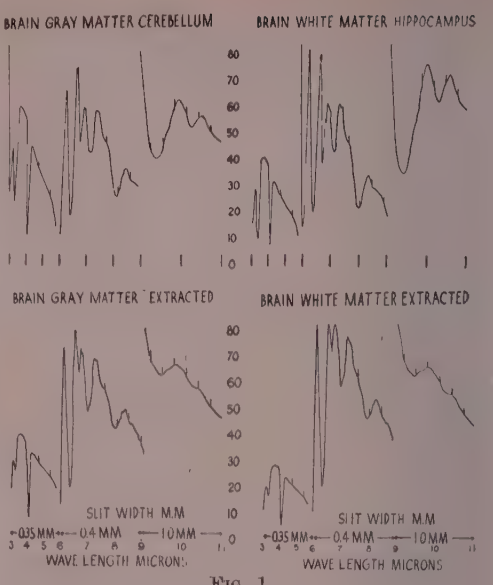

FIG. 1.

extraction experiments similar to those reported by Barer, Cole and Thompson were carried out. Fig. 1 shows the spectra of white and grey matter of the brain before and after extraction with a mixture of ether and chloroform. It is clearly visible from this figure that the bands at 10.27-.30 μ and 9.31-.35 μ, which were so characteristic for the neural tissue, completely disappeared after the extraction, that the unassigned band at 8.06 μ was greatly diminished, and that polyamide vibrations as those at 6.44 μ (deformation vibration of NH) were not influenced by the extraction at all. Barer *et al.*, have already pointed out that their observations (frog nerve tissue) do not necessarily mean that the substances which had been removed by the extraction had to be lipids. They suggested that the reduction of the band at about 9.30 μ was also consistent with the removal of -c-o- bonds such as occur in esters and implied that the band near 8.10 μ which they also found greatly reduced by the extraction appeared to be rather in the polyamides than the lipids.

Quantitative infra red spectroscopy: the effect of insulin on the infra red spectra of brain tissue. The great constancy and reproducibility of infra red spectra of neural

tissues from healthy animals justified an attempt at quantitative estimation of those structures which produce the characteristic spectra of brain tissue of control animals and at comparison of data thus obtained with results of quantitations of brain tissue spectra from animals in insulin shock. The fact that the substances producing the characteristic bands in neural tissue, *viz*: at 10.27-.30 μ and at 9.31-.35 μ could be removed by chloroform ether extraction while the band at 6.44 μ (deformation vibration of N H) was not influenced by the extraction at all, indicated that this band of the polyamide vibrations (6.44 μ) could be used as an internal standard for the quantitative estimation of the substances causing the vibrations at 10.27-.30 μ and at 9.31-.35 μ. It was assumed that the ratio of optical densities $\frac{D\ 10.30}{D\ 6.44}$ called K_1 and $\frac{D\ 9.35}{D\ 6.44}$ referred to as K_2 were independent of the thickness of the sections and the "protein back ground," and that they were true relations of the concentration and the molecular extinction of the structures producing the vibrations at 10.27-.30 and 9.31-.35 μ. The correctness of these assumptions was proved by the fact that sections of the same tissue but of different thickness (25 and 50 μ) and duplicates of the same tissue gave almost identical K_1 and/or K_2 figures. It should be pointed out, however, that measurements and calculations agree with these assumptions only if they are carried out with great accuracy, in bands which are clearly distinguishable. Whenever sharply defined bands are not visible, indicating very small concentrations of these structures, these calculations may give erroneous conclusions, and might better be replaced by well worked out base-line method(8).

In a larger number of sections through different parts of the brain, the K_1 values varied between .21 and .45 and the K_2 values between .47 and .78. This shows greater variation than would actually exist if the same parts of the brain, *e.g.* hippocampus, tectum, cerebellum, and diencephalon were compared. Especially large spectral differences between grey and white matter of the brain were apparent. In one experiment, for example, the grey matter section showed a K_1 of .25 and a K_2 of .57; the white matter gave a K_1 of .31 and a K_2 of .78.

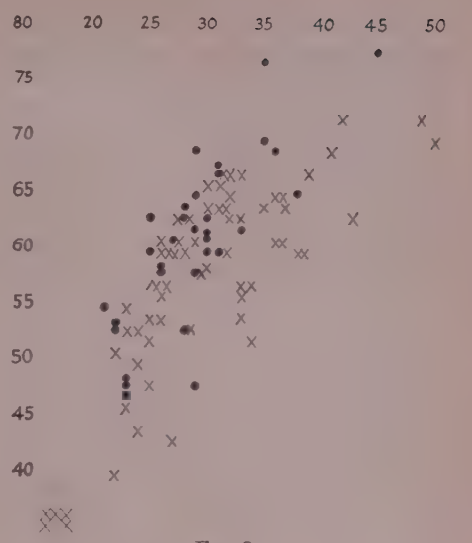

FIG. 2.

The K_1 and K_2 values of brain tissues (hippocampus, tectum, cerebellum, and diencephalon) from 7 control rabbits and 10 rabbits in insulin shock are shown in a distribution graph in which K_1 (abscissa) is plotted against K_2 (ordinate) (Fig. 2). The full dots indicate the figures obtained from the brain tissue of controls and the crosses, those from tissue of animals in insulin shock. In the majority of the experiments (6 out of 10) some of the series of brain sections from animals in insulin shock showed significantly different K values when compared with the K values from normal rabbits. In 5 brain sections of rabbits in insulin shock, the K_1 and/or K_2 figures were either so low that accurate calculation was impossible or the K values were actually zero. Comparison of the actual spectra of the same brain area of a control and a rabbit in insulin shock (Fig. 3) clearly shows these fundamental differ-

8. Wright, N., *Ind. Eng. Chem.*, 1941, v13, 1.

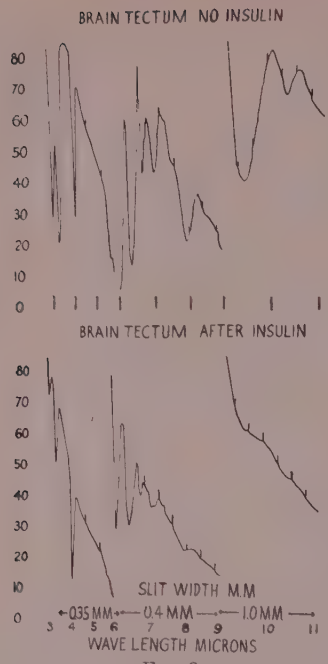

FIG. 3.

ences. In 4 other brain sections of rabbits in insulin shock, the K_1 and K_2 figures were considerably lower than those found in any brain section of control animals. A shift in the relation of K_1 to K_2 occurring in other brain sections from animals in insulin shock is also indicated in the graph and needs further evaluation. In 3 animals free of shock symptoms after insulin injection, sections through different parts of the brain gave about the same K figures as found in control rabbits.

Summary. 1. A method for recording of infra red spectra from frozen tissue section of 25-50 μ thickness and from areas as small as 3 x 3 mm is described. 2. The infra red spectra of tissues show a fingerprint region which is so characteristic of the individual organ that it was frequently possible to identify the tissue from its infra red spectrum. 3. The assignment of tissue spectra to chemical structures as nucleic acid derivatives has been discussed. 4. A method for quantitative infra red spectroscopy in brain tissue has been described. 5. Significant spectroscopic changes in brain tissue of animals in insulin shock have been demonstrated.

Received December 18, 1950. P.S.E.B.M., 1951, v76.

Effect of Proteolytic Enzymes and Fixation on Metachromasia of Skin Collagen. (18459)

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In the course of some histochemical observations on generalized and circumscribed myxedema we(1) have had occasion to treat sections of fixed tissue with certain proteolytic enzymes. We were surprised to find that incubation of sections with pepsin and trypsin led to the appearance of metachromatic staining quality in collagen which ordinarily does not display this property(2). This observation has led us to pursue the subject further in order to compare the effects of these two enzymes on unfixed and fixed preparations.

Methods. Human skin, obtained at autopsy or operation, has been used throughout. Frozen sections were made of unfixed material. Some were treated with crystalline pepsin* or trypsin* in the unfixed state; others were fixed in either neutral formalin or 80% ethanol, after which they were washed and treated with the same enzymes. The enzymes were used in a concentration of 5%; the pepsin was dissolved in acetate buffer, pH 5.0, the trypsin in veronal buffer at pH 7.4.

1. Follis, R. H., Jr., To be published.

2. Wislocki, G. B., Bunting, H., and Dempsey, E. W., *Am. J. Anat.*, 1947, v81, 1.

* Worthington Biochemical Laboratory, Freehold, N. J.

TABLE I. Effect of Incubation With and Without Enzymes on Unfixed and Fixed Collagen.

Treatment	pH	Unfixed	Fixation in 80% ethanol	Fixation in formalin
Pepsin	5.0	Meta.*	Meta.	Meta.
Buffer	5.0	Ortho.†	Ortho.	Ortho.
Trypsin	7.4	Ortho.	Meta.	Meta.
Buffer	7.4	Ortho.	Ortho.	Ortho.

* Meta. = Metachromatic.
† Ortho. = Orthochromatic.

Control sections were incubated in buffer without enzyme. The time of incubation was 2-6 hours. All sections were stained for similar periods with weak toluidine blue.

Results. The results are shown in Table I. When unfixed skin is stained with toluidine blue the collagen takes up very little stain in contrast to the purplish color of the epidermis and appendages. With the concentrations utilized here there was only the slightest blue tint to the connective tissue bundles. In contrast, in unfixed sections which had been digested with pepsin the collagenous fibers exhibited intense metachromasia while those treated in a similar fashion with trypsin did not. On the other hand when frozen sections were fixed in either alcohol or formalin before incubation with the enzymes metachromasia was present after enzymatic digestion with both pepsin and trypsin.

Discussion. We fully realize that metachromatic staining is not a specific histochemical test for the demonstration of mucopolysaccharides in tissue. However, metachromatic dyes such as toluidine blue are useful tools with which to reveal alterations in the chemical nature of certain materials. The physiochemical basis of metachromasia is poorly understood. According to Michaelis (3) certain substrates have the ability to promote polymerization of the dye molecules so that the wave length of the peak absorption of light is shifted. Mucopolysaccharides are among the materials which are capable of doing this.

It is well known that skin contains chrondroitin sulfuric acid, hyaluronic acid and doubtless other mucopolysaccharides(4,5). Despite this, fresh skin collagen is not metachromatic. On the other hand fresh tendon, cartilage and umbilical cord all of which contain these materials are metachromatic. On purely chemical grounds Meyer(6,7) has called attention to the ease with which chrondroitin sulfuric acid may be removed from cartilage, *i.e.* by extraction with $CaCl_2$; from this he has assumed that this mucopolysaccharide is loosely bound to protein. On the other hand much more drastic measures, *i.e.* strong alkali, are necessary to separate this material from skin collagen. One would therefore infer that this mucopolysaccharide is more strongly bound to the protein in skin than that in cartilage.

The unmasking of metachromasia in undenatured skin by pepsin but not by trypsin is of interest because of the well known differences in action of these two enzymes on collagen(8,9). The former is able to digest collagen while the latter is not. It would seem highly suggestive then that pepsin may attack the protein component so as to free groups of the mucopolysaccharide which might then combine with the dye. On the other hand, since trypsin is not able to split the protein, no groups would be available to bind the dye. This is, of course, purely conjectural but may furnish a possible way to study chemically the combination of mucopolysaccharide and protein in collagen.

Summary. When unfixed skin collagen, which ordinarily is only lightly orthochromatic, is treated with pepsin it becomes metachromatic. Trypsin does not have this effect. Collagen denatured by alcohol or formalin fixation is rendered metachromatic by both pepsin and trypsin.

3. Michaelis, L., *Cold Spring Harbor Symposia on Quantitative Biology,* 1947, v12, 131.

4. Meyer, K., and Chaffee, E., *J. Biol. Chem.,* 1941, v138, 491.

5. Watson, E. M., and Pearce, R. H., *Am. J. Clin. Path.,* 1947, v17, 507.

6. Meyer, K., *Am. J. Med.,* 1946, v1, 675.

7. Meyer, K., *Ann. N. Y. Acad. Sc.,* 1950, v52, 961.

8. Sizer, I. W., *Enzymologia,* 1949, v13, 288.

9. Sizer, I. W., *Enzymologia,* 1949, v13, 293.

Received December 18, 1950. P.S.E.B.M., 1951, v76.

Effect of 17-Hydroxy-11-Dehydrocorticosterone and Adrenocorticotropic Hormone upon Plasma Gamma Globulin, Fibrinogen, and Erythrocyte Sedimentation Rate.* (18460)

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(Introduced by G. W. Thorn)

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The use of 17-hydroxy-11-dehydrocorticosterone (Cortisone) in the management of rheumatoid arthritis brought into prominence by Hench and his collaborators(1) and extended to adrenocorticotropic hormone (ACTH) by Thorn and others(2) has stimulated great interest in the mechanism of action of these drugs on various metabolic processes. Studies reported in this communication describe the relationship between 3 of the blood elements which are altered by Cortisone and ACTH treatment, namely, plasma fibrinogen, plasma gamma globulin, and erythrocyte sedimentation rate.

Materials and methods. Clinical material. Observations were made on 12 patients hospitalized for typical severe active rheumatoid arthritis and 3 hospital patients (A.G., A.V., and R.S.) with actively progressing generalized scleroderma. Blood samples were obtained before, during, and after one or more courses of treatment with Cortisone or ACTH. A dose of 100 to 200 mg a day of Cortisone acetate in aqueous suspension‡ administered intramuscularly in divided doses every 6 hours was used. ACTH§ 80 to 100 mg a day was likewise given in divided doses intramuscularly each 6 hours.

Sedimentation rates. 5 ml of blood is collected in a tube containing the dried crystals from 0.2 ml of balanced oxalate(3). The sedimentation rate was determined by the Rourke-Ernstene method(4). The upper limit of normal for this procedure in this laboratory is 0.35 mm fall per minute.

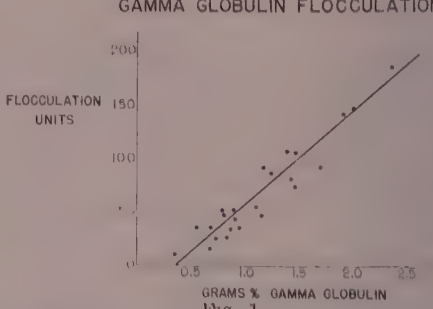

FIG. 1.

Relation between flocculation and electrophoresis methods for the determination of gamma globulin levels in various normal and pathological sera.

Gamma globulin. Kunkel's flocculation method(5) for determining gamma globulins was modified to the extent of doubling the volumes of reagents used and reading the degree of turbidity with the Klett-Summerson colorimeter. Flocculation of 1/10 ml of serum was produced with 6 ml of the zinc sulphate solution described by Kunkel(5). Fig. 1 records 30 random normal and pathological sera on which the gamma globulin content was known from previous electrophoretic analyses. There is a linear relation between the two methods. For the purpose of this study the value of 70 flocculation units has been considered the dividing line between a normal and an abnormal level.

Fibrinogen. Plasma fibrinogen was estimated

* Supported in part by a research grant from the Robert Breck Brigham Hospital, the Asthma Fund, Harvard Medical School, and a U. S. P. H. S. research grant.

† Stanley Tausend Fellow in Medicine.

1. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet. Mayo Clin.*, 1949, v24, 181.

2. Thorn, G. W., Bayles, T. B., Massell, B. F., Forsham, P. H., Hill, S. R., Smith, S., and Warren, J. E., *N.E.J.M.*, 1949, v241, 529.

‡ Merck and Co.

§ Armour Standard LA1A.

3. Ham, T. H., and Curtis, F. C., *Medicine*, 1938, v17, 447.

4. Rourke, M. D., and Ernstene, A. C., *J. Clin. Invest.*, 1930, v8, 545.

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TABLE I. Effect of ACTH and Cortisone on Gamma Globulin, Fibrinogen, and Sedimentation Index.

Patient	Gamma globulin, flocculation units			Fibrinogen, mg/ml			Sedimentation index mm/ml			R
	Start	End	Change	Start	End	Change	Start	End	Change	
H.	80	56	—24	7.6	2.3	—5.3	1.35	.15	—1.20	ACTH
E.	242	183	—59							”
H.A.-1	200	100	—100							”
-2	195	112	—83	4.7	5.5	+0.8	1.90	.50	— .40	”
-3	120	49	—71							”
E.B.-1	135	75	—60	6.0	2.5	—3.5	1.00	.25	— .75	Cortisone
-2	100	60	—40	10.0	4.0	—6.0	1.20	.25	— .95	”
-3	119	65	—54	11.9	4.6	—7.3	1.50	.70	— .80	”
C.-1	130	112	—18	7.2	3.8	—2.4	1.20	1.00	— .20	”
-2	133	100	—33	14.0	7.8	—6.2	1.10	.80	— .30	”
-3	85	98	+13	6.6	6.0	—0.6	1.10	1.00	— .10	”
N.-1	54	40	—14	6.0	10.0	+4.0	0.65	.20	— .45	”
-2	48	42	— 6	8.0	5.0	—3.0	0.85	.30	— .55	”
B.	70	82	+12	8.0	2.6	—6.4	0.65	.35	— .30	ACTH
A.-1	120	66	—54							”
-2	136	53	—83	9.2	5.4	—3.8	1.85	.35	— .50	”
H.B.	65	40	—25	9.0	6.5	—2.5	1.25	.55	— .70	Cortisone
M.	120	50	—70							ACTH
B.	70	30	—41	8.0	3.5	—4.5	0.75	.25	— .50	Cortisone
C.P.	103	67	—36	10.0	7.0	—3.0	0.90	.40	— .50	”
A.G.	36	26	—10							ACTH
A.Z.	135	56	—79	6.6	2.2	—4.4	1.35	.40	— .95	”
R.	117	90	—27							”
Avg	105	67	—38	8.3	4.9	—3.4	1.16	.59	— .57	

as clottable protein after the method of Morrison(6). 4.0 ml of venous blood was discharged into a tube containing 0.75 ml of acid citrate dextrose solution‖ and gently mixed. Plasma was obtained by centrifugation. One or 2 ml of this plasma, depending on the expected level, were made up to 19 ml in 0.85% sodium chloride solution. To this was added 1 ml of a solution containing 50 units of human thrombin.¶ The clot was allowed to retract overnight, separated, washed, dried, and weighed. The normal fibrinogen content of plasma is 2.5-3.0 mg per ml.

Results. Table I presents the values obtained on the 16 patients studied. The average fall during hormone treatment in the gamma globulin flocculation units was from 105 to 67. The average fall in mg per ml of fibrinogen was from 8.3 to 4.9, and finally, the average fall in the sedimentation index was from 1.16 to 0.59 mm per second. These changes are significant. Fig. 2 indicates the scatter of results, each dot representing the change in a single treatment period.

In patient A.G. the figures indicate that treatment suppressed a normal gamma globulin to a subnormal value. Fig. 3 is the plasma electrophoretic pattern on this same patient before and at the end of treatment, showing these same changes pictorially.

Sedimentation indices are affected by many factors, including the plasma concentration of both fibrinogen and gamma globulin. Grey and Mitchell(7) demonstrated that an increase of 0.2 g% of this protein is sufficient to raise the one-hour rate of fall of erythrocytes 300%, while an increase of the gamma globulins of 0.4 g% changes the sedimentation rate 100%. Both of these quantities are well within the range of change in protein concentration that we have described here. In

6. Morrison, P., *J. Am. Chem. Soc.*, 1947, v69, 2723.

‖ 2.20 g % trisodium citrate $2H_2O$
2.20 g % dextrose
0.80 g % citric acid

¶ Kindly supplied by the Department of Physical Chemistry, Harvard Medical School.

7. Grey, S., and Mitchell, E., Proc. Soc. Exp. Biol. and Med., 1942, v51, 403.

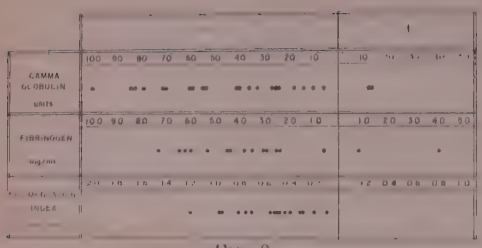

Fig. 2.

The change in gamma globulin, fibrinogen, and sedimentation rate during ACTH or cortisone therapy.

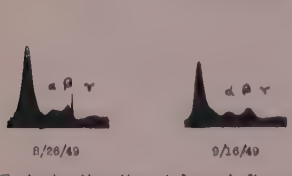

Fig. 3.

The suppression of a normal gamma globulin level to a subnormal value by ACTH.

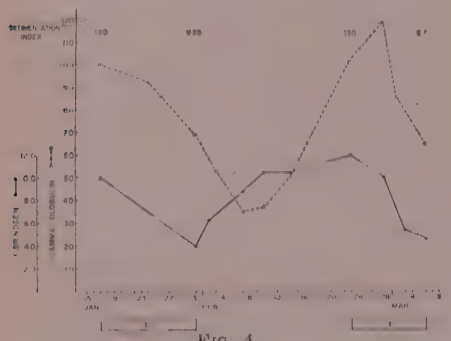

Fig. 4.

The interrelationship between the gamma globulin and the fibrinogen during cortisone therapy in rheumatoid arthritis.

general, fibrinogen concentrations most closely paralleled sedimentation rates, as has been reported by Ham and Curtis(8) for the Rourke-Ernstene method. In patient H.A., however, the fall in sedimentation rate correlated with the fall in gamma globulins, as treatment in this case was accompanied by no significant change in the fibrinogen level. Patient E. V. (Fig. 4) presents the usual pattern of the inter-relationship between these proteins during and after hormone treatment. Fibrinogen levels were more rapidly affected by treatment than were gamma globulin levels. This is easily seen in the period immediately following her first course of treatment, when the gamma globulins continued to fall, while fibrinogen levels promptly rose again. Reciprocal divergences of these values were present during the first week of the second course of treatment, when the gamma globulins continued to rise, while the fibrinogen was already on its way down.

Discussion. It seems likely that the difference in the rates of response to hormone therapy of fibrinogen and gamma globulin levels reflects the rates of synthesis of these two proteins. The best estimate of the rate of gamma globulin turnover was made by Schoenheimer *et al.*(9) who found that the half life of an individual gamma globulin molecule is approximately 2 weeks. Our observation of a one-week delay of the gamma globulin response to hormone therapy would fit in well with these data. Fibrinogen, on the other hand, would seem to be a protein with a very rapid turnover, particularly when it is noted that its absolute level in the serum can change 2- or 3-fold within a few days' time. Hence, the effect of ACTH or Cortisone therapy on this protein could be expected to manifest itself rather promptly.

The role of the adrenal cortical hormones in the lysis of lymphoid tissue is readily recognized as possibly an important factor in lowering the blood gamma globulin(10). An understanding of the effect of the cortical steroids on the fibrinogen, however, must rest with a fuller knowledge of the effect of these hormones upon liver metabolism, as fibrinogen seems to be manufactured by this organ(11).

While it is possible that the changes in the blood protein levels responsible for the sedimentation rate are entirely secondary to variation in disease activity, it is also reason-

8. Ham, T. H., and Curtis, F. C., *Medicine,* 1938, v17, 413.

9. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.,* 1942, v144, 545.

10. Selye, H., *Endocrinology,* 1937, v21, 169.

11. (a) Drury, D. R., and McMaster, P. D., *J. Exp. Med.,* 1929, v50, 569; (b) Jones, T. B., and Smith, H. P., *Am. J. Physiol.,* 1930, v94, 144.

able to assume that they are the result of a direct action of the cortical steroids upon their manufacture. Such an explanation would be consistent with the known "anti-anabolic" properties(12) of these hormones. Therefore, until more data is accumulated on this point, it will be wise to use the sedimentation rate in parallel with the eosinophil count, merely as an index of continued hormonal activity, not as a criterion of effectiveness of therapy on disease activity.

Conclusions. 1. Both ACTH and Cortisone therapy depress the gamma globulin and fibrinogen levels in the blood of patients with active rheumatoid arthritis and scleroderma.

2. Changes in these 2 blood proteins are responsible for the observed changes in the sedimentation rate.

3. At the present state of our knowledge, the sedimentation rate cannot be used as an index of the effectiveness of hormone therapy on the active disease process.

The authors are grateful to Dr. Walter Lever of the Department of Physical Chemistry, Harvard Medical School, for the electrophoretic data reported here.

The technical assistance of Miss Aline Warren and Mr. Jack Kasarjian was greatly appreciated.

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Received December 19, 1950. P.S.E.B.M., 1951, v76.

Fat Necrosis Produced by Exposure of Pancreatic Duct and Duodenal Mucosa.* (18461)

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In previous papers(1,2) the effects of experimental injuries to the pancreas were reported. It had been found that open transsection of the main pancreatic duct or the lengthwise incision of the main duct and its two main branches was not followed by fat necrosis unless the animal had been fed before or after the operation, or when cholinergic drugs had been given postoperatively. Extensive crushing or complete transverse tearing of the pancreas did not result in any intra-abdominal fat necrosis, regardless of whether the animals were fed or starved pre- or postoperatively. In a discussion of these results an experimental procedure was suggested of which no report was found in the pertinent literature. Nevertheless, this paper is not a claim for an original procedure and is merely a report on the use of an effective method for the experimental production of fat necrosis.

Twenty male and female mongrel dogs, weighing from 7 to 12 kg, were subjected to an aseptic operation under pentobarbital-sodium anesthesia. The main pancreatic duct was exposed and carefully excised together with a circular flap of duodenal wall approximately 2 cm in diameter, with the orifice of the duct in its center; the opening in the duodenum was closed. It was possible to perform this procedure with a minimum of contamination. The dogs were fasted pre- and postoperatively, but water was given freely after the operation. Most animals received penicillin and streptomycin postoperatively.

Of these 20 dogs, 3 showed no ill-effects. They were sacrificed on the 3rd, 4th, and 18th day respectively, and there was no evidence of intraperitoneal disease. A fourth dog, which was in good shape and would probably have survived, was sacrificed on the 8th postoperative day, and autopsy revealed a moderate amount of disseminated fat necrosis. In 2 of these 4 dogs, the duodenal flap

* Aided by the Isaac Kate Meyer Fund. The Department is in part supported by the Michael Reese Research Foundation.

1. Popper, H. L., *Surg. Gynec. Obst.*, 1949, v88, 254.

2. Popper, H. L., *Am. J. Dig. Dis.*, 1949, v16, 343.

was covered by firmly adherent omentum, in the other 2 by firmly adherent duodenum.

Of the remaining 16 dogs, 1 died on the 2nd, 8 on the 3rd, 4 on the 4th, and one on the 8th postoperative day. Two dogs appeared to be moribund, and they were sacrificed on the 3rd and 4th postoperative day. All of these dogs showed severe pathologic changes, with varying amounts of hemorrhagic fluid in the peritoneal cavity and most extensive intra-abdominal fat necrosis, involving mostly the omentum, the mesentery, and the parietal and peri-renal subperitoneal tissue. Pancreas and liver were grossly normal. The duodenal flap was usually somewhat cyanotic, but otherwise intact and easily demonstrable. In the majority of dogs fat necrosis was found in the chest also, mostly on the superior surface of the diaphragm, on the pericardium, in the paravertebral region and on the inner surface of the chest wall, usually along the intercostal spaces. No fat necrosis was found in any other region. Bacteriological tests showed no growth of bacteria from the peritoneal fluid in some dogs, while in others various aerobic and anaerobic bacteria were found.

Sixteen of 20 operated animals showed extensive fat necrosis which, if the 2 moribund animals were included, was fatal on the 3rd or 4th postoperative day in most dogs. Three had no intraperitoneal disease and one showed fat necrosis of moderate degree.

The operation described above appears to be the most simple and efficient one of all methods we have employed for producing fat necrosis in the dog, since it shows a high percentage (85%) of positive result with a rather uniform course. Other procedures are less reliable and results are not as uniform. The technic used by Dragstedt *et al.*(3) for testing the toxicity of pancreatic secretion is similar in principle to the method described above. However, a 2-stage operation, an open flap of jejunum, and a rubber tube from the cannulated pancreatic duct onto the jejunal flap, represent 2 separate operations and introduce complicating factors.

Summary. Excision of the pancreatic duct together with a duodenal flap surrounding its papilla is followed in the majority of dogs by extensive intraperitoneal and marked intrathoracic fat necrosis, and usually leads to the death of the dog on the 3rd or 4th postoperative day. This appears to be the most reliable procedure for producing fat necrosis in the dog.

3. Dragstedt, L. R., Haymond, H. E., and Ellis, J. C., *Arch. Surg.*, 1934, v28, 232.

Received December 21, 1950. P.S.E.B.M., 1951, v76.

Distribution and Excretion of Radioactive Hafnium[181] Sodium Mandelate in the Rat. (18462)

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The advent of radioisotope therapy for malignant disease and other pathological conditions has stimulated the investigation of many elements and compounds in the search for substances with tissue or organ specificity, and for an understanding of principles governing biological localization. Element 72, hafnium, has been studied by us in an attempt to determine its distribution in the body after the intravenous administration of its man-

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delate salt. No previous reports regarding the distribution or toxicity of hafnium compounds have been found. Preliminary experiments indicate that the LD_{50} for a 10 day period of hafnium sodium mandelate when given intravenously to white rats is approximately 75 to 100 mg of hafnium metal per kilo of body weight. When administered in daily intraperitoneal doses to white rats as the gluconate salt, the LD_{50} for a 10 day period is about 2.0 to 3.0 g of hafnium metal per kilo of body weight(1). These values are comparable to those obtained for zirconium salts(2,3).

Chemical properties. Hafnium is a member of the carbon-silicon family and occupies a position in the fifth sub-group of elements with titanium, zirconium, and thorium. It is a metal, usually tetravalent, but in some cases divalent, and closely resembles zirconium in its physical and chemical properties although it is more basic(4,5). It has been estimated to be thirtieth in abundance of elements composing the earth's crust(6) and is contained in all zirconium ores in amounts varying up to 30%. Because of this close association with zirconium and the difficulty of separation, a paucity of knowledge exists regarding its properties.

Radioactive hafnium. Hafnium has not been found in any naturally occurring radioactive form, but 5 radioactive isotopes have been artificially produced: Hf^{172}, Hf^{173}, Hf^{175}, Hf^{179}, and Hf^{181}. Of these, Hf^{181} is most readily available in appreciable quantities being easily formed by the bombardment of stable hafnium with thermal neutrons. Hafnium^{181} possesses a half-life of 46 days. Its decay scheme has not been established with certainty, but most likely its transition to tantalum^{181} is accompanied by the emission of one beta ray (0.4 mev) and 5 gamma rays (0.087, 0.13, 0.13, 0.34, and 0.48 mev) (7,8).

Method. Hafnium^{181} oxide (0.900 g) with an estimated activity of 60 mc/g, obtained from the Oak Ridge nuclear reactor, was converted to hafnium tetrachloride by passing chlorine gas and carbon tetrachloride vapor over it at a temperature of 550-600°C. Immersion of the tetrachloride into approximately 250 ml of water formed hafnium oxychloride. This was evaporated to 50 ml. A 10 ml aliquot was then placed in a volumetric flask containing 0.550 g of mandelic acid and a drop of cresol blue indicator. Sodium hydroxide and water were added until a pH of 7.0 and a volume of 25 ml were reached. All procedures were done with necessary precautions for the safe handling of radioactive material. One-half ml of the radioactive hafnium sodium mandelate solution (containing about 3.06 mg of hafnium metal) was injected intravenously into the tail vein of 17 healthy, white, male, inbred, Wistar-stock rats weighing between 250-350 g. These animals were kept in metabolism cages where urine and feces could be collected separately. Daily urinary and fecal samples were taken for analyses with the exception of the first, when 8 hour specimen collections were made, and for the last 4 days when 48 hour samples were obtained.

The animals were sacrificed in groups of 3 at one, 2, 3, 4, 8, and 16 day intervals (only 2 animals comprised the 8 day study). They were lightly anesthetized with an intraperitoneal injection of nembutal and a sample of blood was aspirated from the inferior vena cava. This was subsequently centrifuged and divided into red cell and plasma portions.

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3. Cochran, K. W., Doull, J., Mazur, M., and DuBois, K. B., *Arch. Ind. Hyg. and Occup. Med.*, 1950, v1, 637.

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8. Chart of the nuclides, General Electric Co., 1950.

TABLE I. Urinary and Fecal Excretion of Intravenously Administered Hafnium[181] Sodium Mandelate Expressed as % of Total Activity.*†

8 h.	16 h.	1 d.	2 d.	3 d.	4 d.	5 d.	6 d.	7 d.	8 d.	10 d.	12 d.	14 d.	16 d
						Urine							
2.09	.39	.18	.27	.30	.18	.10	.11	.28	.18	.28	.20	.17	.19
‡	2.48	2.66	2.93	3.23	3.41	3.51	3.62	3.90	4.03	4.31	4.51	4.68	4.87
						Feces							
.02	.19	.22	.60	.36	.16	.06	.13	.07	.03	.04	.06	.08	.04
§	.21	.43	1.03	1.39	1.55	1.61	1.74	1.81	1.84	1.88	1.94	2.02	2.06
						Cumulative total excretion							
2.11	2.69	3.09	3.96	4.62	4.96	5.12	5.36	5.71	5.87	6.11	6.45	6.70	6.93

* Avg of 3 animals for each time period except 2 animals for the 8-day study.

† % total activity equals counts per min. for specimen divided by total counts per min. of entire animal plus excretions.

‡ Cumulative urinary excretion.

§ Cumulative fecal excretion.

After the blood had been obtained, the rat was viviperfused with isotonic saline. The animal was then dissected and the following specimens, or aliquot parts, taken for analysis: liver, spleen, pancreas, salivary glands, kidneys, adrenals, testes, urinary bladder, heart, lungs, thymus, thyroid, brain, eyes, trachea, incisor teeth, incisor pulp, prostate, red cells, plasma, muscle, pituitary, bone (femoral shaft, femoral epiphyses, and mandible), stomach, stomach contents, small intestine, small intestinal contents, cecum, cecal contents, large intestine, large intestine contents, tail, and carcass. The tissues were weighed and then gently heated with concentrated nitric acid until dissolution occurred. After this pseudo-ashing, a 10 ml sample was placed in a Petri dish for measurement of the radioactivity using a lead-walled, vertical, counting chamber with a TCG-2, thin mica window, Geiger-Mueller tube. The inside bottom of the dish was 1.6 cm from the tube window. The same geometry was employed for all counting. Radioactivity of the urine was determined by counting an aliquot portion after sulfuric acid digestion of the filter paper on which the urine had been absorbed. The feces was homogenized by incubation with a concentrated papain solution and an aliquot sample counted.

Total blood weight was assumed to be 7% of the total body weight(9). From this and the hematocrit, the plasma and red cell masses were determined. Skeletal weight was calculated as 7% of the total body weight(10). Muscle weight was assumed to be carcass minus skeletal weight. The per cent of the total activity of the animal and excretions in the various organs and body systems was calculated from the radioactivity measurements, after correction to a zero time. Concentration values, or specific activity, for the different tissues were derived and expressed as per cent per gram adjusted to a standard body weight.

Results. The excretion of hafnium[181] sodium mandelate is given by Table I and is illustrated by Fig. 1 in terms of per cent total activity. The daily urinary, daily fecal, cumulative urinary, cumulative fecal, and total cumulative excretion have been tabulated.

Table II shows the per cent of total activity retained in the most important organs and body systems. This was derived from the counts per minute of the structure involved divided by the number of counts per minute in the entire animal and its excretions, with the exception of its tail. The amount of activity in an animal's tail probably represented unabsorbed hafnium[181] and therefore was not included.

Table III lists the concentration values, or specific activity, in the same organs. This was obtained by dividing the per cent total activity by the weight in grams of the organ or body system with each animal corrected to a standard body weight.

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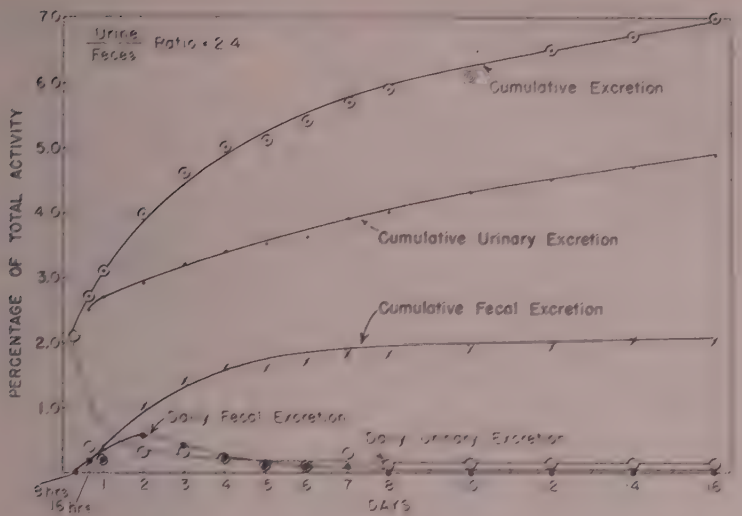

EXCRETION OF INTRAVENOUS Hf^{181} SODIUM MANDELATE

FIG. 1.

Graph illustrating the urinary, fecal, and total excretion of hafnium[181] sodium mandelate for a 16-day period following its intravenous administration to the white rat.

Discussion. After the intravenous administration of hafnium[181] sodium mandelate, the hafnium was slowly excreted, only 7% having been eliminated at the end of 16 days. Of this, 5% was excreted within the first 4 days. A larger portion was removed by the kidneys, the urine/feces ratio being 2.4 in this study. Between 70 and 90% of the total activity was accounted for by the liver, spleen, the skeletal, muscular, and integumentary systems in each of the time intervals studied. The liver and spleen respectively showed highest retention on an organ basis. Slight, but appreciable amounts were found in the adrenals, thyroid, pancreas, salivary glands, and testes. Little, or no activity was contained in the brain, eyes, pituitary gland, teeth, heart, lungs, thymus, trachea, and prostate. The gastro-intestinal tract in general contained small amounts and an occasional high determination was believed to reflect the difficulty in separation of contents from the wall rather than any absorption.

Appreciable amounts of hafnium[181] remained in the blood for the first 3 days, after which only 0.1-0.5% of the total activity was present. At the end of 24 hours, 4% of the total activity was in the blood. In each determination, about 95% of the activity was found in the plasma, with little or none in the red cells.

Expressed as specific activity (Table III) the spleen exhibited the greatest value followed by liver, bone, and kidneys respectively. The pelt which contained a high percentage activity during the first 2 days showed a

TABLE II. Distribution* of Hafnium[181] Sodium Mandelate in Organs Expressed in % of Total Activity.†

Organ	5 hr	24 hr	2 days	4 days	8 days	16 days
Liver	19.8	41.3	36.5	37.8	42.8	44.9
Spleen	3.9	9.1	11.8	12.5	7.7	2.1
Bone	13.8	13.0	21.5	15.4	25.8	12.5
Kidneys	2.2	1.4	0.8	1.5	2.1	1.6
Blood	4.0	1.0	0.5	0.2	0.1	0.1
Heart	0.2	0.3	0.3	0.1	0.2	0.2
Lungs	0.7	1.3	1.3	0.4	0.5	0.3
Pelt	14.4	3.2	3.0	3.2	1.6	0.8

* Each column represents avg determinations in 3 animals with exception of 8-day period, when 2 animals were studied.

† % total activity equals organ counts per min. divided by total counts in entire animal and excreta.

TABLE III. Distribution* of Hafnium^{181} Sodium Mandelate in Organs Expressed in % per g Corrected to a Standard Body Weight.

Organ	24 hr	48 hr	72 hr	96 hr	8 days	16 days
Liver	1.8	4.9	4.3	4.3	6.4	6.5
Spleen	5.5	9.4	19.2	23.8	17.4	6.3
Bone	0.8	0.8	1.2	0.9	1.5	0.6
Kidneys	1.5	0.8	0.4	0.8	1.3	0.9
Blood	0.5	0.1	†	†	†	†
Heart	0.3	0.3	0.3	0.1	0.2	0.2
Lungs	0.7	0.7	0.8	0.2	0.2	0.2
Pelt	0.2	0.1	†	†	†	†

* Each column represents avg determinations in 3 animals with exception of 8-day period, when 2 animals were studied.
† Negligible.

marked decline subsequently. Tissue autoradiograms confirm these findings and will be discussed in more detail later(11).

These data indicate that hafnium^{181} sodium mandelate has little specific localization in the body to recommend its consideration for radiation therapy of individual organs or tissues.

11. Boyd, G. E., Unpublished observations.

Conclusions. 1. The distribution and excretion of radioactive hafnium^{181} sodium mandelate after its intravenous administration was studied in 17 rats sacrificed at intervals of 1, 2, 3, 4, 8, and 16 days. 2. Seventy to 90% of the total activity was retained in the liver, spleen, the skeletal, muscular, and integumentary (pelt) systems. 3. Specific activities showed the spleen to be highest, followed by the liver, bone, and kidneys. 4. The adrenals, thyroid, pancreas, salivary glands, and testes showed a slight, but appreciable retention. 5. Hafnium^{181} in the blood was found chiefly (95%) in the plasma and remained in appreciable amounts to 4 days. 6. Seven per cent of the total dose was excreted in 16 days with more being eliminated in the urine than in the feces, the urine/feces ratio being 2.4.

The authors wish to express their appreciation for the generous technical assistance of Drs. W. C. Davis and Boyd Weaver, Plant Y-12, Oak Ridge, Tenn.

Received December 21, 1950. P.S.E.B.M., 1951, v76.

Failure of a Flavonoid to Reduce Radiation Mortality in Mice. (18463)

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Various investigators have reported flavonoids to be beneficial in the treatment of animals after exposure of the whole body to potentially lethal amounts of radiation(1-3). Sokoloff *et al.*(3) have adequately discussed the beneficial reports. Other reports(4,5) have stressed the ineffectiveness of rutin. Further discussion of the rationale behind the use of these compounds is out of the scope of this paper which reports another failure.

Materials and methods. A flavonoid compound isolated from citrus fruit was used.* For injections the material was dissolved in saline (0.9%), pH adjusted to 7.0 and diluted so that 1 ml contained 6.6 mg of flavonoid. The flavonoid was started 5 days before ir-

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2. Clark, W. G., Uncapher, R. P., and Jordan, M. L., *Science*, 1948, v108, 629.
3. Sokoloff, B., Redd, J. B., and Dutcher, R., *Science*, 1950, v112, 122.
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5. Kohn, H. I., Robinett, P. W., and Cupp, M. N., Atomic Energy Commission Document 2176 (13p), 1948. Technical Information Division, Oak Ridge, Tenn.

* Supplied by Dr. Boris Sokoloff, Florida Southern College, Lakeland, Florida, and reported to contain Quercitin-like substance 4%, eriodictin 15%, chalcone hesperidin-glucose hesperidin 80%, and calcium phosphorus ash 0.38%.

TABLE I. Mortality Data of Flavonoid Treated and Control Irradiated Mice. (32 mice in each group.)

Dose of x-ray	Days after x-ray →	No. of mice dying per day																	Total dying during period	% mort. in this exp.	% mort.* this strain mice
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	30			
1050 r	Treated		5	11	11	3	2	0											32	100	
	Untreated	1	2	4	2	3	5	2	2	5	6								32	100	99.6
950 r	Treated		3	4	10	8	5	1	1	0									32	100	
	Untreated		2	3	4	6	2	1	8	5	0	0	0	1					32	100	96
850 r	Treated		1	6	8	7	4	4	2	0									32	100	
	Untreated			1	3	1	4	4	4	6	3	3	0	0	1	0	0	0	30	94	88
750 r	Treated				3	5	3	6	8	0									25	78	
	Untreated		1	3	1	1	1	3	1	5	3	2	3	1	1	0	1		27	84	64
0	Treated																		0	0	
	Untreated																		0	0	

* Based on repeated mortality studies on total of over 5000 mice during period of June 1947 to June 1950.

radiation and continued until the mice died or for 15 days after irradiation. It was given 2 times per day subcutaneously. The total daily dose was 0.13 mg/g of mouse. White, Swiss, inbred, male mice weighing 20-26 grams were irradiated simultaneously with the radial beam of the 2.0 mev GE industrial x-ray machine(6). The radiation factors are: 2000 KVP, 1.5 ma, no added filter, 15.0 (±0.15r) r per minute in air at 2 meters, HVL 4.3 mm P^b. Mice were given the following doses of x-ray: 750, 850, 950, and 1050 r. Sixty-four mice were exposed at each dose. Thirty-two mice were treated and 32 were not treated with flavonoid. In addition, 30 mice were treated with the flavonoid, but were not irradiated.

Results. In Table I the results are tabulated. At the doses of radiation used the compound gave no protection. The control, non-irradiated, injected mice gained weight at the same rate as did the non-injected control mice. There was no evidence of any toxicity of the compound in mice at the dose used.

Discussion. The dose of flavonoid used was at least 4 times that per gram of animal used by Sokoloff *et al.*(3), in their experiments on rats that demonstrated an apparent protective action.

The doses of radiation used in these experiments varied from approximately an LD_{60} to almost an LD_{100} for this strain of mouse. In this dose range almost 100% of the mice dying after the 5th day have extensive purpuric hemorrhages throughout their organs. If, as reported by Field(1), Clark(2), and Sokoloff, *et al.*(3), the flavonoids protect against radiation by preventing hemorrhage through protection of the capillary bed, one might reasonably expect these compounds to protect mice at doses of radiation where hemorrhage is prevalent. The current experiments do not support this concept.

A summary of the data previously reported shows protection in dogs(1), rats(3), and guinea pigs(2), and no protection in rats(5) and mice(4). In some of the reports(1-3) it is not clear whether the treated and untreated mice were irradiated simultaneously or paired in some manner to assure that the control and treated groups are really comparable. Ample experience at this Institute with radiation lethality experiments has demonstrated the absolute necessity of simultaneous irradiation of paired control and treated animals.

It is our present opinion that these compounds have little, if any, protective effect against radiation. However, with the possibility of atomic war, the need of a non-

6. Chambers, F. W., Jr., Morgan, J. E., and Istock, E. T., Report 26, Project NM 006 012.08, 7 Dec. 1949, Naval Med. Research Inst., Bethesda, Md.

toxic compound that will be effective as a prophylactic and as a therapeutic agent is so great that further studies should be made with the flavonoid group of compounds on a wide spectrum of animals to make certain that no possibility of a beneficial effect is overlooked.

Conclusion. 1. A flavonoid (vit. "P") compound is of no value in the treatment of radiation injury in the LD_{60} to LD_{100} range in mice.

Received December 26, 1950. P.S.E.B.M., 1951, v76.

The Effect of Aureomycin on Digestion in Steers. (18464)

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Experiments with several species of animals have demonstrated that aureomycin and other antibiotics have a pronounced effect on microorganisms in the gastrointestinal tract. The ruminant is primarily dependent upon an active microbial flora for efficient digestive processes. An experiment was undertaken, therefore, to determine the effect of aureomycin* on the digestion of feed by steers.

Experimental. Six Hereford steers weighing about 620 pounds were used in this digestion experiment. The steers were kept in metabolism stalls with provisions made for separate collection of feces and urine. They were fed a constant amount of ration during 10-day preliminary and 15-day collection periods. Digestibility of nutrients and nitrogen retention were calculated from the chemical analyses of feeds and excreta. Blood samples were taken periodically for the determination of blood urea. All other experimental technics were similar to those reported by Briggs and Gallup(1). The composition of the daily ration fed each steer during collection periods is given in Table I. The digestibility of these rations without the addition of aureomycin had been determined with these same steers in a previous series of digestion trials. In the previous experiment, each of the steers was fed the basal ration and the urea† ration during 10-day preliminary and 10-day collection periods. No feed refusals or digestive disturbances were observed during this entire period of approximately four months.

Prior to the present experiment it was necessary to obtain information on the approximate amount of aureomycin which would not interfere with continued feed consumption. A level of 0.6 g per day was found to produce a marked anorexia and a severe fetid diarrhea within 48 to 72 hours. This condition persisted for 4 to 5 days after aureomycin feeding was discontinued. The anorexia appeared to be due to a digestive disturbance and not to unpalatability of the ration. The feeding of 0.2 g per day did not appear initially to interfere with a constant daily feed intake. Aureomycin feeding was discontinued until the appetite and digestive processes appeared normal. A standard 10-day preliminary period was then conducted during which each steer was fed a constant amount of the assigned ration without aureomycin. Aureomycin (0.2 g) was then added to the rations of steers 1, 2, 3, and 6. Steers 4 and 5 were continued on the basal ration. Urine and feces collections were started immediately.

Results and discussion. The digestion coefficients, nitrogen retention, and blood urea levels determined in the previous experiment during which the steers were fed the rations without aureomycin are given in part A of

* The crystalline aureomycin hydrochloride was supplied by Dr. E. L. R. Stokstad, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

1. Briggs, H. M., and Gallup, W. D., *J. Ani. Sci.*, 1949, v8, 479.

† Urea in the form of "Two-Sixty-Two" feed compound was supplied by E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

TABLE I. Composition of Daily Ration (grams).

Ingredients	Steer number and ration identification					
	1 Urea + aureomycin	2 Basal + aureomycin	3 Basal + aureomycin	4 Basal	5 Basal	6 Urea + aureomycin
Prairie hay	1800	1800	1800	1800	1800	1800
Corn	1730	1730	1730	1730	1730	1730
Soybean oil meal	70	70	70	70	70	70
Urea	45	—	—	—	—	45
Bone meal	50	50	50	50	50	50
Salt	30	30	30	30	30	30
Aureomycin	0.2	0.2	0.2	—	—	0.2

TABLE II. Effect of Aureomycin on Digestion Coefficients, Nitrogen Retention, and Blood Urea.

Steer No.	3-day period	% apparent digestibility of					N retention, g	Blood urea, mg %
		Dry matter	Crude protein	Ether ext.	Crude fiber	N-free ext.		
(A) Avg values obtained in previous exp.								
				Basal ration				
All		64.94	48.32	65.43	65.49	69.65	7.62	8.41
				Urea ration				
All		65.45	62.40	66.62	65.55	69.70	12.14	20.06
(B) Avg values obtained in aureomycin exp.								
				Basal ration				
4	1	69.07	51.93	63.31	66.35	74.91	9.10	7.66
and	2	68.38	52.68	58.58	62.77	74.93	7.67	6.59
5	3	70.69	55.42	65.94	67.01	76.20	9.74	8.86
	4	69.37	55.15	66.93	65.45	75.40	10.27	7.10
	5	72.67	60.83	71.52	70.60	77.96	13.03	5.34
	Avg	70.04	53.20	65.26	66.44	75.88	9.96	7.11
				Basal ration + aureomycin				
3	1	76.38	65.64	74.63	66.29	81.86	17.74	15.43
	2	63.71	56.27	62.76	49.71	70.48	9.63	14.24
	3	58.00	47.60	66.31	35.88	67.25	9.60	13.41
	4	54.06	45.31	60.50	30.72	63.49	1.66	20.38
				Urea ration + aureomycin				
1	1	74.58	72.10	71.58	69.98	79.65	14.38	21.11
and	2	66.23	67.46	62.77	52.70	73.52	6.79	22.06
6	3	63.84	65.05	65.44	51.37	70.20	6.46	22.63
	4	59.53	68.42	67.57	39.78	66.79	6.96	23.78
	5	59.58	67.86	65.13	42.86	66.89	9.04	22.24

Table II. The same values for consecutive 3-day periods following the addition of aureomycin to the rations are given in part B of Table II. The third day after aureomycin feeding was started steers 1 and 2 developed a mild anorexia and diarrhea. Steer 2 continued to refuse feed and had to be removed from the experiment. About the fifth day, steers 1, 3, and 6 became slightly constipated and developed a "paunchy" or bloated condition. Their feces became dry and fibrous. During the last three days of the experiment, steer 3 developed such a severe fetid diarrhea and marked anorexia that digestion coefficients were not calculated for this final 3-day period. Steers 4 and 5 consumed their rations and no digestive disturbance was noted.

Data in Table II show that the most pronounced effect of aureomycin was on the digestibility of crude fiber, which suggests that it had a detrimental effect on the cellulytic micro-organisms in the gastrointestinal tract. In periods 4 and 5 the digestibility of dry matter was decreased to less than 60 percent and crude fiber digestibility was decreased to less than 45 percent. Aureomycin also decreased the digestibility of dry matter and nitrogen-free extract. Steers fed aureo-

mycin had higher blood urea levels than when aureomycin was not fed.

Summary. The effect of feeding crystalline aureomycin hydrochloride on digestion and nitrogen retention was determined in balance trials with six steers. Feeding 0.2 g aureomycin daily caused a marked reduction in the digestibility of crude fiber. It also reduced the digestibility of dry matter and of nitrogen-free extract. When 0.6 g of aureomycin was fed daily to steers, it produced a marked anorexia and a fetid diarrhea within 48 to 72 hours. This condition persisted for several days after the aureomycin feeding was discontinued. Continued feeding of 0.2 g aureomycin daily, produced somewhat milder digestive disturbances.

Received December 26, 1950. P.S.E.B.M., 1951, v76.

Loss of Protection by Vaccination Following Cortisone Treatment in Mice with Experimentally Induced Tuberculosis. (18465)

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Cortisone administered in single or repeated doses has been shown to reduce the amount of circulating antibody(1-3). Cortisone has also been reported to decrease the tuberculin reactivity of guinea pigs sensitized to the tubercle bacillus(4,5) and to cause a spread of the disease in guinea pigs(6). Also studies in the mouse, using both a chronic and acute tuberculosis infection showed that the enhancing effect of cortisone was most marked in the chronic infection(7). In the present study, in addition to acute and chronic infection, the effect of cortisone on mice vaccinated with heat-killed cells was also investigated. Our results are in essential agreement with those of D'Arcy Hart and Rees(7) and in addition indicated that the protective effect of vaccination is abolished by cortisone.

Materials and methods. 1. *Strain of organisms.* Two substrains of *Mycobacterium tuberculosis,* human type, H37Rv, were used. The first, possessing the original characteristics of the strain as obtained from the Trudeau Laboratories, was highly virulent for mice. The second, obtained after 2 years of serial weekly passage in Dubos Tween-albumin medium, showed reduced virulence for mice. The highly virulent substrain has been maintained by serial passage in mice. 2. *Preparation of vaccine and vaccination of mice.* Vaccine was prepared from the original strain grown in Dubos Tween-albumin medium for 14 days at 37°C. The culture was adjusted to a turbidity permitting 65% transmission of light at 650 mμ* and subjected to free-flowing steam for one hour. Sterility controls of such suspensions showed no growth after 2 months of incubation at 37°C. Mice† weighing 12 g were injected intraperitoneally with 0.5 ml amounts of vaccine administered 3 times per week for a period of 2 weeks. 3. *Infection of animals.* Test animals were infected by intravenous inoculation of 0.25 ml amounts of culture in Dubos medium adjusted to permit 70% transmission of light at 650 mμ. Mice dying during the course of the

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*On a Coleman Spectrophotometer Model 6A.

† Barckman, IS-32 mice were used throughout the course of these experiments.

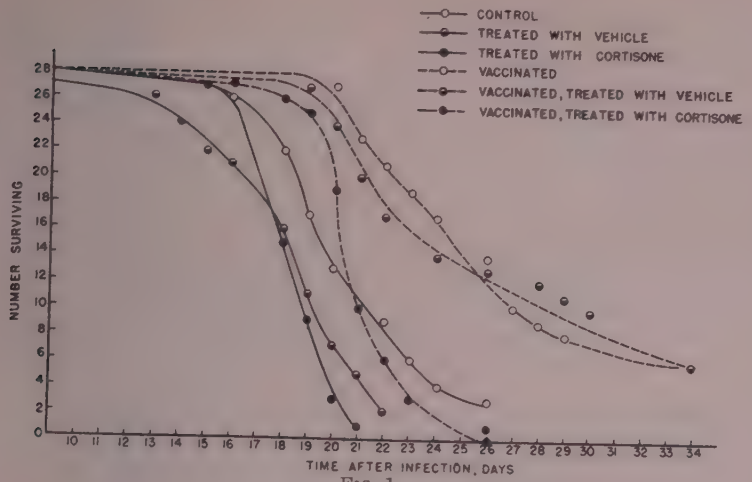

FIG. 1.
Effect of cortisone on tuberculosis in vaccinated mice.

experiment as well as those surviving the tests were autopsied and examined to confirm tuberculosis as the cause of death. 4. *Drug administration.* Mice were injected once daily with 0.1 mg of cortisone acetate‡ available as a five-fold aqueous dilution of a stock containing 5 mg of cortisone acetate per ml of vehicle. The vehicle contained 0.4% Tween 80, 0.5% sodium carboxymethyl cellulose, 1.5% benzyl alcohol and 0.9 sodium chloride.

Experimental. The data of a typical experiment are presented graphically in Fig. 1. Six groups of 28 mice each were used. Groups 1, 2 and 3 were not vaccinated; 4, 5 and 6 were vaccinated. All mice were challenged with culture of the virulent substrain 2 days after vaccination. Animals in groups 2 and 5 were treated with 0.1 mg of cortisone administered once daily 6 days per week beginning on the day following infection and those in groups 3 and 6 were treated with the same amount of vehicle as used for the cortisone treated animals. The 2 remaining groups, 1 and 4 served as untreated controls. The experiment was terminated 34 days after infection.

Among the non-vaccinated animals, Group 1, the untreated controls showed an average survival time of 19.7 days. Treatment with vehicle or cortisone reduced the average survival time approximately 1½ days. Vaccination prolonged survival time to 25 days and this prolongation of survival time was not significantly affected by treatment with vehicle alone. However, treatment of vaccinated mice with cortisone depressed the survival time to 20.5 days a figure approaching that observed for the non-vaccinated controls. To confirm tuberculosis as the cause of death, lungs from a majority of the mice in each group were examined histologically and in addition spleens, livers and kidneys from a smaller number of animals were examined. After staining by Hematoxylin-Eosin, Ziehl-Nielsen and Gram, sections were examined microscopically. The numerous lesions in the lungs and the minimal ones in the other tissues showed many acid fast rods unaccompanied by other organisms. Thus in non-vaccinated animals, cortisone had no effect on the outcome of the rapidly fatal infection that could not be explained by a slightly toxic effect of the vehicle in the presence of infection. Under these conditions the animals may have had insufficient time to develop a significant degree of immunity. When however, animals were protected by vaccination cortisone overcame the advantage conferred by vaccination.

2. *Effect of cortisone on infection of low*

‡ Cortone Merck a brand of Cortisone Acetate was used in these experiments.

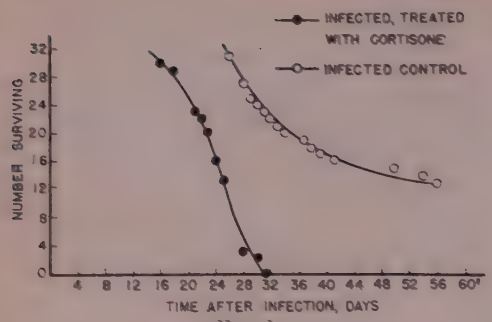

FIG. 2.

Effect of cortisone on low virulence tuberculosis in mice.

virulence. The effect of cortisone on an infection of reduced virulence was studied in two trials. In the first 2 groups of 32 mice each were infected with the substrain of reduced virulence. The mice in one group were treated with 0.1 mg of cortisone administered subcutaneously once daily 6 days per week and the other group served as the infected controls. The data for this test, which was continued for 56 days, are shown graphically in Fig. 2. The median survival time for the infected controls was 40 days while treatment with cortisone reduced the average survival time to 24 days. The untreated controls began to die on the 25th day and at the termination of the experiment on the 56th day 40% were still alive. The cortisone treated animals began to die on the 17th day and all were dead by the 31st day.

In the second trial groups of 32 mice each again were used and the experiment was continued for 35 days. None of the infected control animals died within the experimental period whereas only 50% of the animals treated with cortisone survived.

Discussion. Enhancement of tuberculosis during the hyperadrenal state has been observed in man and experimental animals. The mechanisms by which cortisone enhances the infectious process in experimental tuberculosis have not yet been completely elucidated. The results of the present study suggest that one of the mechanisms by which cortisone may alter the course of this disease is by interference with the immune response. In rapidly progressive tuberculosis where the role of acquired immunity would be expected to be minimal, cortisone did not exert a significant effect on the course of the disease. However, when vaccinated animals were treated with cortisone, the prolonged survival conferred by vaccination was lost.

The results obtained with slowly progressive tuberculosis are in harmony with the hypothesis that cortisone affects the immune response. The slowly progressive disease would permit the animal to acquire immunity. Here the enhancing effect of cortisone on the progress of the infectious process was highly significant; in one of the experiments presented, all the cortisone-treated animals were dead by the 32nd day, whereas more than 40% of the infected controls were still alive at the 56th day after infection.

This interpretation of the present findings is consistent with observations indicating that treatment with cortisone suppresses the amount of circulating antibody.

The data presented are in agreement with the observations of D'Arcy Hart and Rees that cortisone markedly exacerbated chronic tuberculosis infection in the mouse but are not completely in accord with their findings that cortisone treatment enhanced the acute type of infection. This difference may be related to the high dose of cortisone employed by these investigators, 0.5 mg as compared with 0.1 mg used in the present study.

Summary. 1. Treatment with cortisone overcame the beneficial effect of vaccination in mice infected with a highly virulent strain of *M. tuberculosis*, human type, but did not significantly enhance the lethality in non-vaccinated mice. 2. Treatment with cortisone increased the susceptibility of mice to infection with *M. tuberculosis* of reduced virulence.

Received December 27, 1950. P.S.E.B.M., 1951, v76.

Influence of Cortisone on Free Hydroxyproline in the Developing Chick Embryo. (18466)

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In the course of the chromatographic examination of the free amino acids and peptides of the tissues of the developing chick embryo, determinations were also made on extracts prepared from tissues of embryos which had received single injections of 1 mg of cortisone (cortisone acetate-Merck) onto the chorioallantoic membrane. The cortisone-treated embryos were tested because of the marked and specific inhibition in their growth produced by the hormone(1). The most consistent difference observed between the tissues of the normal and cortisone-injected embryos was a large increase in the content of free hydroxyproline in the latter. It is the purpose of this communication to report some of the details of this finding.

Experimental. Embryos and preparation of tissues. The tissues were obtained from 3 batches of fertile eggs. The first 2 groups were from a sex-linked Barred Rock stock obtained during the summer of 1950 in Bar Harbor, and the third was from White Leghorns obtained in New York City in the fall of the same year. The eggs were incubated at 38°C and 75% relative humidity and were kept in one position, being moved once daily when candled for viability. The injections of cortisone were made onto the chorioallantoic membrane in the manner previously described(1). The injections were made on the eighth day, since the cortisone-effect appears to be initiated between the eighth and tenth days, even when the hormone is given earlier(1). In the third set of eggs a control group was also injected with Δ^1-17 α-hydroxy progesterone, a steroid not possessing any cortisone-like activity. In the first and third set of eggs the embryos were sacrificed at 18 days of age and then weighed and examined grossly for the typical effects produced by cortisone(1). In the second group the surviving embryos were sacrificed at various earlier times as well as at 18 days. The effect of the cortisone on growth and feather formation was considerably less in the Barred Rock embryos than in the White Leghorns. The growth rate of the normal embryos of the latter strain was also generally slower. Extracts of the freshly excised tissues were prepared for analysis essentially as previously described(2) with the exception that the salt was removed from a number of the samples by electrolytic desalting(3).

Chromatographic and chemical procedures. Aliquots corresponding to 75 mg of fresh tissue were placed on paper for 2-dimensional paper chromatography by the conventional methods(4,5). In several instances larger aliquots were employed to detect substances present only in small amounts. Care was taken to run the extracts of the comparable control and cortisone-treated embryos through the entire preparative and chromatographic procedure at the same time, so that slight

* Supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service, and the Charles F. Kettering Foundation.

† We wish to acknowledge the aid of the American Cancer Society, the National Cancer Institute of the United States Public Health Service, the Damon Runyon Memorial Fund for Cancer Research, and the Babe Ruth Cancer Fund of the American Cancer Society.

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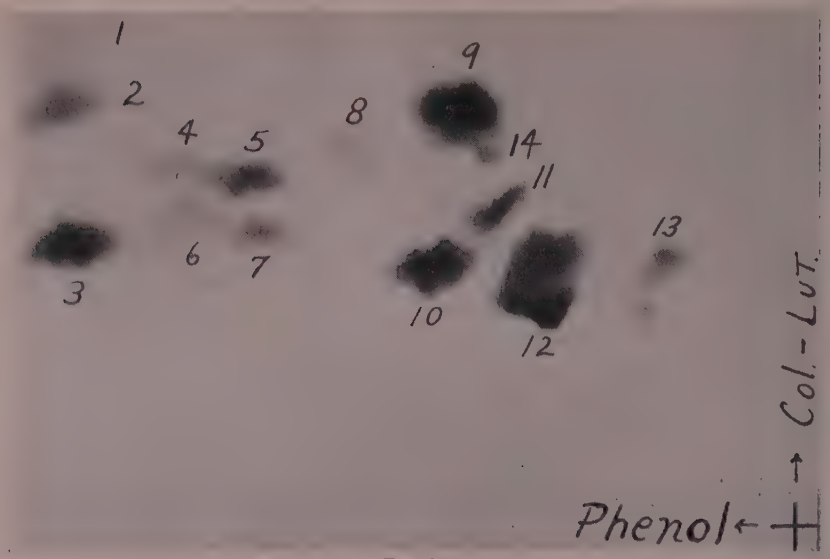

FIG. 1

Chromatogram of extract of 150 mg of brain of 18-day cortisone-injected chick embryo. Key to numbers on chromatograms: Leucines, 1; valine, 2; γ-aminobutyric acid, 3; hydroxyproline, 4; alanine, 5; β-alanine and/or citrulline, 6; glutamine, 7; threonine, 8; taurine, 9; glycine, 10; serine, 11; glutamic acid, 12; aspartic acid, 13; unknown substance, 14.

variations in conditions would not influence the results. In some instances it was possible to analyze the extracts for hydroxyproline content by a recently devised colorimetric procedure(6).

Chromatographic results. In all cases chromatograms of the extracts of brains, livers, and hearts of 14, 16, and 18-day embryos showed notable increases in the content of free hydroxyproline in the cortisone-injected animals within each experimental set as compared with the same tissue of the comparable control. The identity of the hydroxyproline was established by the characteristic orange-brown color, the position on the chromatogram, and the complete superimposition of the spots formed when pure hydroxyproline was added to the tissue extracts. In a number of cases, but not in all, the tissues of the cortisone-treated chicks also seemed to contain somewhat more free glycine than the controls. There were no consistently observable differences in the concentrations of the other ninhydrin-reactive constituents. In the tissues taken from 9 through 12 days the patterns of free amino acids were virtually identical in the normal and injected embryos.

In Fig. 1 and 2 are shown chromatograms from comparable aliquots of desalted extracts of brain from pooled samples of 18-day embryos with and without cortisone treatment. Photographs in Fig. 1 and 2 were taken with a blue filter in order to accentuate the color due to hydroxyproline. The spot produced by hydroxyproline (spot 4) was too light in the case of the control tissue to be reproduced on the photograph. It is obvious that the content of this amino acid is higher in the cortisone-injected embryos. In most cases the chromatograms were virtually indistinguishable except for the intensity of spot 4 and sometimes spot 10 (glycine). Although occasionally some of the other ninhydrin-reactive constituents were present in somewhat larger amounts in the tissues of either the normal or cortisone-treated embryos, these were not consistent observations. The original chromatograms were shown in pairs to several individuals not di-

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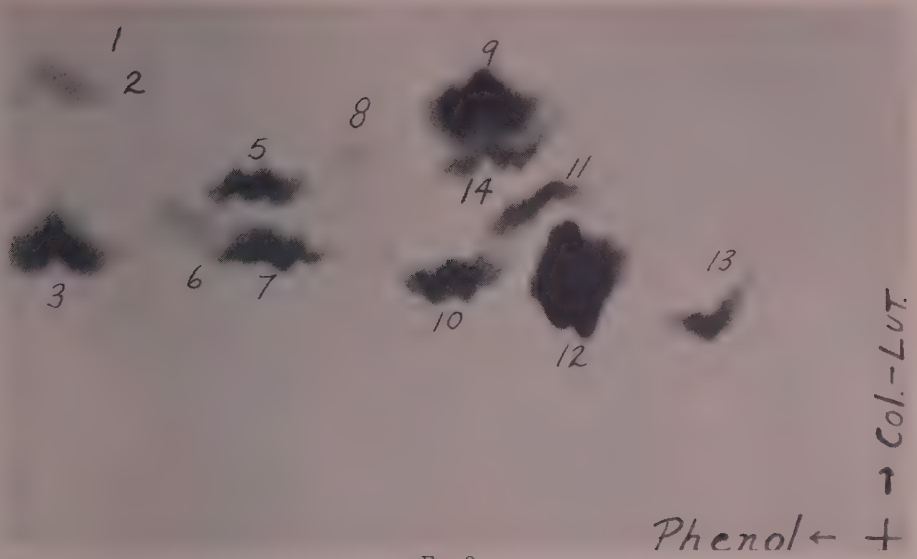

FIG. 2.
Chromatogram of extract of 150 mg of brain of 18-day control chick embryo.

rectly concerned with the experiments, and they were asked to separate them into two groups on the basis of the intensity of the hydroxyproline spot. Employing this criterion within each experimental group they were able to separate all of the chromatograms from the tissues of the cortisone-treated embryos from those of the same tissue of the controls of the same age without a single error.

Colorimetric determination of hydroxyproline content. While the present experiments were in progress a colorimetric procedure for the determination of hydroxyproline became available(6). Sufficient quantities of some of the frozen extracts were left to enable us to perform the analyses shown in Table I. It is seen that in each series the tissues of the cortisone-treated embryos contained more hydroxyproline than did those of the comparable controls. The effect was most marked in the brain. Δ^1-17 α-hydroxy progesterone seemed to depress the level of hydroxyproline slightly. The embryos of the second series showed less effect of the cortisone than did the other two groups as judged by the inhibition of growth and feather formation. In this group the levels of free hydroxyproline were lower both in the controls and in the cortisone-treated embryos than in the third series. However, the *relative* increases in hydroxyproline were approximately the same in both groups.

Discussion. Hydroxyproline was not detected in 5 purified egg proteins(7) and was also absent from the egg white and yolk of the unincubated egg(8). It has been shown to accumulate in the embryo and embryonic membranes as development proceeds, the first traces of this amino acid becoming detectable in the embryo after the fourth day(8). The results of the latter investigator also suggest that the hydroxyproline of the embryo is present principally in the form of collagen or collagen-like substances. The present investigation shows that the only consistent abnormality in the pattern of free amino acids of the tissues of the developing chick embryo produced by the injection of cortisone was a marked increase in the content of free hydroxyproline. This suggests the possibility that there may be a specific effect of cortisone on the metabolism of hydroxyproline. On the other hand, this increase in free hydroxy-

7. Lewis, J. C., Snell, N. S., Hirschmann, D. J., and Fraenkel-Conrat, H., *J. Biol. Chem.*, 1950, v186, 23.

8. Neuman, R. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 37.

TABLE I. Effect of Cortisone on Content of Free Hydroxyproline in the Tissues of 18-Day Chick Embryos.

Series	Treatment	Hydroxyproline content; γ per 100 mg of fresh tissue		
		Brain	Liver	Heart
1	Control	12.0	—	—
	Cortisone	68.0	—	—
2	Control	4.0	3.8	5.6
	Cortisone	15.9	9.8	11.3
3	Control	12.0	10.0	12.4
	Inactive steroid*	10.4	6.4	8.4
	Cortisone	50.2	26.4	32.8

* Δ^1-17 α-hydroxy progesterone.

proline may only indirectly reflect the inhibition of the synthesis of collagen or collagen-precursors.

Summary. A chromatographic examination was made of the free amino acids of tissues of normal and cortisone-injected chick embryos. The content of free hydroxyproline was increased markedly in the tissues of the cortisone-treated embryos. The results were confirmed by a colorimetric procedure. With the exception of glycine, in which increases were noted in some instances, the other amino acids showed no consistent changes as a result of the injection of cortisone. The injection of Δ^1-17 α-hydroxy progesterone produced slight decreases in free hydroxyproline content.

Received January 2, 1951. P.S.E.B.M., 1951, v76.

Absorption and Excretion of Viomycin in Humans.* (18467)

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(Introduced by David P. Barr)

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Viomycin, a derivative of a species of *Streptomyces,* is a compound which has been found to have antimicrobial activity for a number of species of bacteria *in vitro* and to protect mice against experimental infections (1,2). It has been of particular interest because of an inhibitory effect upon mycobacteria, including *M. tuberculosis,* and because of its ability to suppress tuberculosis in animals(3). Preliminary clinical studies with viomycin in tuberculous patients have been carried out(4). The present study concerns the absorption and excretion of viomycin in human subjects. In addition, serum concentrations in patients receiving maintenance therapy and concentrations in the cerebrospinal fluid have been determined.

Materials and methods. Preparation of drug. Viomycin‡ was supplied as the highly purified sulfate salt in 1.0 g vials and was dissolved in sterile distilled water in a concentration of 250 to 500 mg per ml for intramuscular injection.

Collection of specimens. In the studies of absorption and excretion after a single intramuscular dose, specimens of blood were drawn under sterile precautions at prescribed inter-

* This study was aided in part by grants from Charles Pfizer and Co., N. Y.; the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

† Bowen-Brooks Fellow, The New York Academy of Medicine; formerly Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service.

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3. Steenken, W., Jr., and Wolinsky, E., *Am. Rev. Tuberc.,* 1951, v63, 30.

4. Werner, C. A., Tompsett, R., Muschenheim, C., and McDermott, W., *Am. Rev. Tuberc.,* 1951, v63, 49.

‡ Supplied by Charles Pfizer and Co., Brooklyn, N. Y.

vals, and the total 24-hour output of urine following the administration of drug was collected in fractional portions. The blood was allowed to clot in the refrigerator at 4°C, and the serum was removed following centrifugation. Aliquots of the urine fractions were Seitz-filtered immediately after collection had been completed. Specimens of blood for serum assay were withdrawn at random from patients receiving daily maintenance doses of viomycin parenterally, and simultaneous withdrawals of blood and cerebrospinal fluid for assay were made in certain subjects. All specimens of serum, urine, and cerebrospinal fluid were kept frozen until time of assay.

Assay technic. Microbiologic assay of the sera was carried out by means of a tube-dilution method previously described(5). With this technic a preliminary 1:4 dilution was made of the unknown serum in broth. Following this dilution, subsequent dilutions were made in 25% pooled human serum broth. When the broth inoculum of culture was added, the final concentration of serum in each tube became 20%. The final dilutions of the unknown were 1:5, 1:6.6, 1:8, 1:10, etc. This method obviated the effect which varying concentrations of serum proteins may exert in the assay procedure. In assaying the urine and cerebrospinal fluid specimens a conventional technic of 2-fold serial dilutions in broth was utilized. In addition, because of expected low concentrations of drug in the specimens of cerebrospinal fluid, further dilutions of 8:10, 7:10, and 6:10 were made of these specimens in broth. The test-organism employed was a strain of *Klebsiella pneumoniae,* type A-D, and the final concentration was a $1:10^6$ dilution of 18 hour growth at 37°C in infusion broth. All assays were read after 18 to 24 hours incubation at 37°C. A standard solution of viomycin in serum or in broth was diluted, inoculated, and incubated concurrently with each series of unknown specimens. The end-point was taken to be the first tube of each series in which macroscopically visible growth could not be detected. All concen-

TABLE I. Serum Concentrations and Data for Urinary Excretion of Viomycin in 5 Subjects Following a Single Intramuscular Dose.

Subject	Dose, mg/kg	Serum conc., μg/ml					Urine conc., μg/ml					% dose recovered				
		½	1	2	4	8	24 hr	0-1	1-2	2-4	4-8	8-23	23-24 hr	1	2	4	8	24 hr
1	52	100	125	125	100	25	<12	100	12500	625	800	100	25	3	28	49	69	72
2	50	125	100	100	62	16	<12	1240	1240	2480	800	200	100	11	28	55	68	77
3	47	62	62	82	33	12	<12	2480	1240	1240	800	200	200	10	32	56	77	91
4	35	60	120	75	30	<15	<15		3170*	5000	800	200	50		18	42	58	65
5	25	100	100	75	20	<15	<15	3170	5000	3170	800	100	<6	25	47	73	95	105

* 0-2 hr specimen.

5. Tompsett, R., Shultz, S., and McDermott, W., *J. Bact.,* 1947, v53, 581.

trations were expressed in terms of pure viomycin base having a potency of 1000 μg per mg.

Results. Absorption and excretion. A single intramuscular dose of viomycin was administered to each of 5 adult males. Three of the subjects received 3 g, and the other 2 received 2 g and 1 g respectively. On a weight basis these doses represented from 25 to 50 mg per kilo. The data concerning the serum and urine concentrations which were achieved and the amounts of viomycin which were recovered in the urine following the single injections of drug have been presented in Table I. The curves of the serum concentrations of 3 subjects which had received doses of 50 mg per kilo have been shown in Fig. 1 together with the mean values for the cumulative urinary excretion of the drug in all 5 subjects. From Table I and Fig. 1 it may be seen that the highest serum concentrations were attained within one-half to 2 hours after injection and ranged from 82 to 125 μg per ml. Thereafter the values diminished, and no drug could be detected in the sera which were obtained 24 hours after injection. The smallest concentration of viomycin which could be measured in the serum by this method was 7.5 μg per ml. Urinary excretion of the drug commenced within an hour after injection, and concentrations of viomycin up to 12 mg per ml were achieved in the urine. Four of the 5 subjects had measurable concentrations of viomycin in the urine specimen which was collected between 23 and 24 hours after the initial dose. Concentrations of viomycin in the urine of less than 6 μg per ml could not be detected with the assay method. The period of greatest urinary excretion of the drug was between 2 and 4 hours after injection. From 65 to 100% of the administered dose of viomycin was recovered in the urine within 24 hours after the single dose had been given (Table I and Fig. 1).

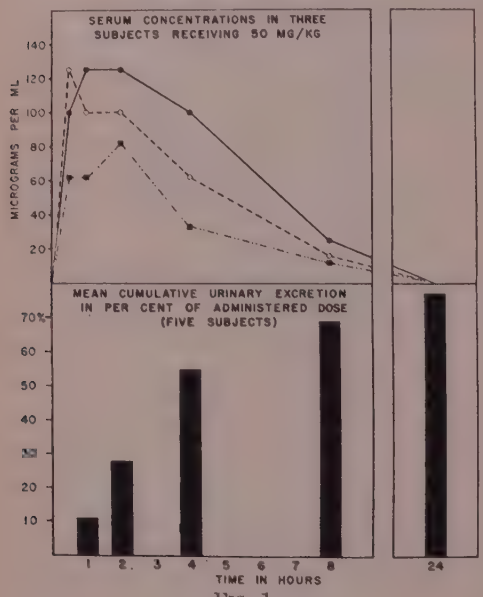

FIG. 1.

Absorption and excretion of viomycin following a single intramuscular dose.

Serum concentrations during maintenance therapy. Determinations of viomycin were made in 22 sera obtained from 6 patients with pulmonary tuberculosis who were receiving daily maintenance doses of 1 g 2 or 3 times daily intramuscularly. These doses represented total amounts of the drug of 50 to 72 mg per kg per day, and the sera were obtained after 14 to 53 days of continuous therapy. In 8 of 14 sera which were obtained 12 hours after the last preceding dose of viomycin no drug was measured, while in the remaining 6 specimens concentrations of 7.5 to 30 μg per ml were detected. In each of 8 specimens obtained 4 hours after the last preceding dose, concentrations of 30 to 100 μg per ml were measured.

Concentrations in cerebrospinal fluid. In single specimens of cerebrospinal fluid obtained from 2 subjects 2 hours after a single intramuscular dose of 3 g, concentrations of viomycin of 10 and less than 4 μg per ml were obtained, while the corresponding serum concentrations were 100 and 125 μg per ml respectively. In a third specimen of cerebrospinal fluid obtained after 60 days from a patient who had received 1 g of viomycin intramuscularly 3 times daily continuously during this time, the concentration of viomycin was 12.5 μg per ml, while the concentration in the serum specimen which had been obtained simultaneously was 100 μg per ml.

None of these subjects had evidence of meningeal involvement.

Comment. The results of this study, which has demonstrated that viomycin was rapidly absorbed and that high concentrations were achieved in the serum after intramuscular injection, may bear only a very loose relationship to the successful utilization of the drug in antimicrobial therapy. It has been encouraging to note, however, that the serum concentrations of viomycin which were attained in the present study were well in excess of the minimal inhibitory concentrations of viomycin required *in vitro* to suppress strains of *M. tuberculosis* isolated from patients with active tuberculosis(4). The pattern of absorption and urinary excretion of viomycin was similar to that of streptomycin, and the serum concentrations which were attained following the intramuscular administration of viomycin have compared favorably with the concentrations of streptomycin which have been achieved following comparable doses of the latter drug(6).

Summary. A study has been made of the absorption and excretion of viomycin following intramuscular administration in humans. Maximum serum concentrations of 82 to 125 μg per ml were attained within 2 hours after a single dose of 25 to 50 mg per kilo. Reasonably high concentrations were achieved in the serum of patients receiving daily maintenance doses of the drug. Small concentrations of viomycin, 10 to 12.5 μg per ml, were detected in the cerebrospinal fluid in the presence of high serum concentrations of the drug, 100 to 125 μg per ml. Viomycin was excreted in the urine in high concentrations, and from 65 to 100% of the administered dose was recovered in the urine within 24 hours.

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Received January 2, 1951. P.S.E.B.M., 1951, v76.

Abnormalities of the Eye Occurring in Young Vitamin E-Deficient Rats. (18468)

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In the course of some studies with vit. E-deficient rats, unusual abnormalities of the eye were observed in the young of mothers deficient in this vitamin. Such lesions had not been encountered before in this laboratory, and have not been mentioned by other investigators to our knowledge.

Although the data are limited, it seems of importance to report them, in view of the recently published studies of Owens and Owens(1) indicating that vitamin E may play an important role in the prophylaxis of retrolental fibroplasia, a disorder which appears frequently during the early months of life in small, prematurely born infants. This condition has been recognized for many years, and has been ascribed to a variety of etiological factors; however, Owens and Owens (2) are the first investigators to follow the progress of the disease from the initial stages of dilatation of the retinal arteries and formation of fibrous bands extending into the vitreous to the final fusing of these bands with folds of the swollen retina to form the characteristic membrane behind the lens. The membrane may be complete, causing total blindness or it may be partial, allowing some vision to be retained. Symptoms accompanying this condition noted by these and other workers(3) were eyeballs smaller than normal and hemorrhages behind the lens. Once the

1. Owens, W. C., and Owens, E. U., *Am. J. Ophth.*, 1949, v33, 1631.

2. Owens, W. C., and Owens, E. U., *Am. J. Ophth.*, 1949, v32, 1.

3. Krause, A. C., *Arch. Ophth.*, 1946, v36, 387.

TABLE I. Summary of Data on Abnormalities of the Eye in Young Rats from Vitamin E-Deficient Mothers.

Maternal history				
Rat No.	Started E-free diet at, days	Litter No.	Age at birth, days	Eye abnormalities of young
1079	14	1	85	2 young weaned, both with small eyes; 1 young, eyes unopened
1199	7	1	75	Large blood clot behind pupil in 1 young
1427	14	1	84	Small eyes in all young (6) of litter; died before weaning
		2	141	White membrane behind pupil in 1 young; small eyes in 1 young; 5 young weaned
1518	7	1	72	White membrane behind pupil in 1 young of 5 weaned
1792	7	3	176	Eyes of all young (4) unopened at weaning
2083	7	1	71	Small eyes in 1 young of 7 weaned
2086	7	1	73	Small eyes in 2 young of 7 weaned

changes occur, they are irreversible, but Owens and Owens have been successful in preventing their appearance almost entirely in a series of premature infants by the administration of dl alpha tocopheryl acetate, starting soon after birth.

In our experiments, female rats that had had access to a vit. E-deficient diet* from either 7 or 14 days of age, and had been weaned at 21 days to the same deficient diet, were mated with normal males as soon as the vagina was patent and regular oestrus cycles had been established. On the fifth day after sperm were found in the vaginal smear, a single dose of 3.0 mg alpha tocopherol acetate (Merck) in olive oil was given. With this treatment, the majority of these animals, when they were from 70-90 days of age, gave birth to first litters of normal size and weight; many of the young died before the 21st day, and the incidence of paralysis was high in the young weaned. The paralyses, which were often severe, were of both the flaccid and spastic type, and affected either fore or hind limbs, rarely both in the same animal. Subsequent matings of the same females, employing the same dose of tocopherol, resulted in a very high percentage of resorptions or in litters consisting of only a few animals.

It was also observed that the eyes of a number of the paralyzed young exhibited abnormalities which suggested the symptomatology of retrolental fibroplasia, as it has been described. These symptoms in the rats were: Eyeballs smaller than normal, eyelids which failed to open, an opaque white membrane visible behind the pupil, and in one instance a large clot of blood completely filling the pupil. These observations, together with data about the mother which have a bearing on these observations, are summarized in Table I. Unfortunately, it was impossible to follow the studies further at the time and to make a systematic histological study of both the abnormal eyes and eyes which appeared to be normal in littermates of these animals; such a study would have revealed beyond a doubt whether or not we were dealing with a disease in rats similar to human retrolental fibroplasia.

Owens and Owens(1) have pointed out that the large amounts of vit. A with which the diet of the premature infant is supplemented routinely may reduce the available vit. E in the digestive tract, since the tocopherols, by virtue of their antioxidant property, serve to protect vitamin A from destruction in the intestine. The diet is already limited in vit. E because of its low fat content. Increased use of water-miscible vit. A preparations has been demonstrated by Kinsey and Zacharias(4) to show significant correlation with increased incidence

* Casein 24%, dry brewer's yeast 8%, Osborne and Mendel salt mixture 4%, lard 10%, cod liver oil 2%, corn starch 52%.

of retrolental fibroplasia.

In this connection, it is of interest that two water-miscible preparations of vit. A (the alcohol and acetate) have proven, in our laboratory, to be less stable than solutions of the same two isomers in cottonseed oil. Both the oil and water-miscible preparations were secured from the same laboratory at the same time and stored together, unopened, at 0°C. After 18 months, vitamin A bioassays demonstrated that the vit. A potency of the oil solutions was unimpaired, whereas the water solutions had lost so much vit. A activity that even when fed at a level of 3.0 units per day (estimated from original potency), the depleted animals with few exceptions either died or lost weight during the 4-week assay period. These data suggest that vitamin A is protected from oxidation to a lesser degree in an aqueous medium than in a cottonseed oil medium, and for this reason may render a correspondingly larger amount of vit. E in the digestive tract unavailable for metabolic processes in the body.

These observations are presented with the thought that studies to prevent the occurrence of retrolental fibroplasia in premature infants might be facilitated by further experimentation with vitamin E-deficient rats to develop a standardized technic for the production of a similar abnormality in young rats.

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Received January 3, 1951. P.S.E.B.M., 1951, v76.

Effect of Dilantin and Mesantoin on the Giant Axon of the Squid.* (18469)

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The effectiveness of dilantin (sodium diphenyl hydantoinate) and mesantoin (sodium phenyl ethyl methyl hydantoinate) in raising cortical thresholds to electric convulsive shocks(1) and their successful applications in the therapy of convulsive disorders are yet unexplained in terms of mechanism. The present experiments employing the isolated giant axon of squid are an attempt to study the actions of these compounds under simplified conditions and to approach an understanding of their mode of action.

Procedure. The giant axon of squid *(Loligo pealii)* was dissected in connection with its ganglion. The nerve was kept for ½ hour in a solution of artificial sea water to attain ionic equilibrium(2), then it was exposed to various solutions and the electrical signs were examined periodically. In other experiments the ganglion was removed and the nerve was threaded through a small bore polystyrene tube into which the electrodes had been sealed as described previously(3). The nerve was then perfused continuously and its electrical activity was recorded on a dual beam cathode ray oscilloscope. Stimulation was delivered by a dual square wave monophasic stimulator. The results of the two procedures were similar. A reduction in the content of both calcium and magnesium of the sea water produced spontaneous firing of the giant axon for long periods without showing electrical signs of injury. Omitting either calcium or magnesium did not produce an adequate condition, since the fibers lost their excitability after a brief period of hyperirritability. The concentration of dilantin or mesantoin in the

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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2. Bullock, T. H., Nachmansohn, D., and Rothenberg, M. A., *J. Neurophysiol.*, 1946, v9, 9.

3. Bullock, T. H., Grundfest, H., Nachmansohn, D., and Rothenberg, M. A., *J. Neurophysiol.*, 1947, v10, 63.

artificial sea water was about 0.6 mg per ml. The insolubility of these compounds makes it difficult to estimate accurately how much remained in solution during the course of the entire experiment. The solutions were adjusted to the usual pH of sea water, *i.e.* pH 8.0-8.3. The penetration of dilantin into the interior of the axon was studied by exposing the fiber to dilantin labelled with N^{15}.† After one hour of exposure the axoplasm of the nerve was extruded and the N^{15} determined in a manner similar to that described previously(4).‡

Results and discussion. a. *Effect on the fiber.* Exposure of the giant axon for ½ to 2 hours to solutions of dilantin and mesantoin produced no appreciable effect on its electrical activity. Although a moderate increase in threshold was noted, it did not differ significantly from the controls. However, in another way an effect of dilantin and mesantoin has been obtained. Reducing the concentrations of calcium in the artificial sea water from .012 M to .006 M and of magnesium from .024 M to .012 M results after 10-15 minutes of exposure in spontaneous firing which persists during the period of perfusion with the sea water deficient in ions. This increased irritability is reversed within 10 to 15 minutes by re-exposing the axon to sea water containing calcium and magnesium in normal concentrations. A similar decrease in irritability has been observed within 2 to 3 minutes by the addition of dilantin or mesantoin to the sea water deficient in ions. After the spontaneous activity ceases, further soaking in the solutions containing dilantin or mesantoin and a reduced concentration of calcium and magnesium causes a reduction in the size of the action potential induced by stimulation and in 5 to 10 minutes its disappearance. However, excitability may be restored by washing the axon in sea water of normal composition. The entire cycle can be reproduced several times with the same axon. The action potential decreases and finally disappears in a way which has no special features.

The results are of interest in view of the application of dilantin and mesantoin to convulsive disorders. Apparently they do not demonstrably affect the normal fiber. Nevertheless, when the physiological equilibrium has been altered and a state of hyperirritability produced, as in the experiments described, these compounds are effective.

b. *Penetration into the axon.* By labelling dilantin with N^{15} the penetration of the compound into the interior of the axon may be determined. The presence or absence of dilantin within the fiber is an important consideration for the problem of its site of action. The nerves were exposed to 0.5 mg dilantin per ml for one hour. The atom per cent excess N^{15} of the dilantin was 28.5%. In the 80 mg of axoplasm collected the atom per cent excess was 1.2%. On the basis of these data the internal axonal concentration of dilantin was 85 per cent of that in the external medium. Since the concentration of dilantin of the outside solution does not remain constant because of a tendency to precipitate during the course of the experiment, the value of 85% is an approximation which may be regarded as conservatively low. It is more likely that equilibrium between the interior of the axon and the outside medium actually was attained during the period of exposure. The rate of penetration of dilantin is relatively rapid. Its entry, for example, is many times faster than that of glycine, alanine and aspartic acid (5). The observation classifies dilantin among those other compounds which have been shown to enter the interior of the nerve fiber (6). Thus far it would appear that only compounds which penetrate affect the conductive process, although mechanisms of their action may be different.

Summary. An action of dilantin and mesantoin on the nerve fiber is described. The

† We are greatly obliged to Dr. George Rieveschl of Parke, Davis & Co. for providing us with N^{15} labelled dilantin.

4. Rothenberg, M. A., Sprinson, D. B., and Nachmansohn, D., *J. Neurophysiol.*, 1948, v11, 111.

‡ We are indebted to Dr. David Rittenberg for the mass spectrographic determinations of N^{15}.

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6. Nachmansohn, D., *Biochim. et Biophys. Acta*, 1950, v4, 78.

addition of these compounds to a perfusion of artificial sea water does not affect the electrical activity of isolated giant axons. In the presence of reduced calcium and magnesium spontaneous firing of the fiber occurs. This may be reversed by returning the nerve to sea water with normal concentrations of calcium and magnesium. A decrease in hyperirritability may also be obtained by adding dilantin or mesantoin to water deficient in calcium and magnesium. Continued exposure to the latter solution reversibly abolishes signs of electrical activity. Dilantin labelled with N^{15} was found to penetrate into the interior of the giant axon of squid. The concentration in the axoplasm of the fiber was close to equilibrium within one hour.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Effect of Histamine and Antihistaminics on Coagulation of Normal and Heparinized Rabbit Plasma. (18470)

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Haley and Harris(1) recently reported that several antihistaminic compounds significantly decreased the coagulation time in both normal and roentgen ray irradiated guinea pigs. This indicated that the antihistaminic compounds were able to inactivate the circulating anticoagulant (heparin?) found in the blood of animals subjected to whole body ionizing radiation(2). However, when one considers the chemical structure of the antihistaminics in relation to other compounds shown to be heparin inactivators both chemically and in a coagulation system(3,4), it becomes difficult to explain the antiheparin activity of the antihistaminics. The structure of these drugs, with the exception of the phenothiazine derivatives, bears no resemblance to the nuclear structure (phenazine, thiazine or oxazine) previously shown to be essential for heparin inactivation(3). Furthermore, the essential primary amine group is absent and the antihistaminics have a tertiary or cyclized amine group in its place. It was these considerations which prompted our *in vitro* investigation of the effect of both histamine and the antihistaminics upon the coagulation of normal and heparinized rabbit plasma.

Experimental. Blood was obtained from 5 rabbits by cardiac puncture every 2 weeks. The pooled blood was mixed with 3.8% citrate at a ratio of 1:4, then centrifuged and the supernatant plasma removed and stored at 4°C until used. All the plasma was filtered through glass wool before use and no plasma was used which was more than 4 days old. The method and chemicals used for studying the effects of histamine and the antihistamines on coagulation were the same as previously described for the dyes(4). Histamine and the antihistamines were dissolved in physiological saline to give final concentrations of 10, 25, 50, 100, 250, 500, 750, and 1000 μg per 0.1 cc. Six separate determinations were made with each concentration of drug, with and without heparin 0.1 μg (0.12 Toronto Units) in the system. The following antihistaminic drugs were investigated: diphenhydramine, tripelennamine, phenazoline, pyranisamine, thonzylamine, phenindamine, Tagathen, metaphenilene,

* This article is based on work performed under Contract No. At-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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4. Haley, T. J., and Stolarsky, F., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 103.

TABLE I. Typical Effects of Antihistamines on Rabbit Plasma Coagulation.

Drug	Plasma	Control value	Conc. in μg/0.1 cc 10	25	50	100	250	500	750	1000
Tripelennamine	Normal	66*	65	65	67	65	70	67	70	67
	Heparinized	221	213	220	205	216	201	158	131	108
Phenindamine	Normal	60	60	60	64	69	71	106	212	276
	Heparinized	227	218	208	204	165	86	109	213	274
Thonzylamine	Normal	59	60	59	59	57	57	57	56	57
	Heparinized	174	170	163	162	167	170	158	147	150

* Avg values of coagulation times in seconds.

Doxylamine, Phenergan, methapyrilene, Thenfadil, Chloroprophenpyridamine, Di-Paralene, chlorocyclizine, pyrathiazine, Anthallan, p-Fluorobenzyl D.P.E., P.D. Co. AH-853, 194-B, Ambodryl, Bristol C-5581H, No. 204 and Foralamin. The Beckman pH-meter was used to determine the pH values of both histamine and the antihistamines at their highest concentration (1000 μg/0.1 cc). Control evaluations using hydrochloric, fumaric, maleic and succinic acids were made at the following pH's: 2, 3, 4, 5, and 6.

Results. The protocol of typical experiments showing the effects of the various antihistamines on normal and heparinized rabbit plasma is given in Table I. Fig. 1 illustrates the typical patterns of response obtained with the various antihistaminics when they are grouped according to the effect produced. The heparin inactivation observed at the lower doses is probably due to the alkaline nature of the antihistaminics and their combination with the acidic heparin molecule. Control evaluations with the various acids showed that the coagulation system, with or without heparin, was unaffected except at pH 2. At this value the systems became incoagulable except in the case of hydrochloric acid which caused the plasma proteins to precipitate.

Comparison of the effects of the various compounds upon normal plasma reveals that histamine, Anthallan, chlorocyclizine, Di-Paralene, Foralamin, Ambodryl, Phenergan, Tagathen, C-5581H and phenindamine have critical levels above which they act as anticoagulants. However, such is not the case with pyrathiazine, 204, PD-AH-853, p-Fluorobenzyl D.P.E., 194-B, methaphenilene, pyranisamine, tripelannamine, phenazoline, diphenhydramine, chloroprophenpyridamine, Doxylamine, Thenfadil, methapyrilene, and thonzylamine. The latter 3 compounds are unique among the compounds investigated in that they have no effect on either normal or heparinized plasma. Pyrathiazine, phenazoline, 204, diphenyhydramine, methaphenilene, P.D. AH-853, 194-B, p-Fluorobenzyl D.P.E., tripelennamine and pyranisamine have critical levels for exerting their maximum antiheparin action and above those concentrations no further inactivation results. Critical heparin inactivation concentrations, above which an anticoagulant effect is observed, occur with histamine, Anthallan, Ambodryl, chlorocyclizine, Di-Paralene, chloroprophenpyridamine, Phenergan, doxylamine, C-5581H, Tagathen, Foralamin and phenindamine.

Discussion. Our results indicate that both histamine and the antihistaminic drugs, with the exception of Thenfadil, methapyrilene and thonzylamine, are capable of inactivating heparin but the quantities required for complete inactivation can only be attained at toxic levels insofar as human dosage is concerned. The inactivation of heparin may be due to the chemical combination of the basic antihistaminic molecule and the acidic heparin molecule. However, one should not overlook the possibility that both histamine and the antihistamines could increase platelet disintegrative activity producing a more rapid release of the platelet thromboplastin, such as has been postulated by Butler *et al.*(5). His results with platelet-free and platelet-rich dog plasma of animals receiving increasing doses of histamine show a similarity to the results

5. Butler, S., Hall, F. R., and Sanford, H. N., *J. Lab. Clin. Med.*, 1950, v36, 710.

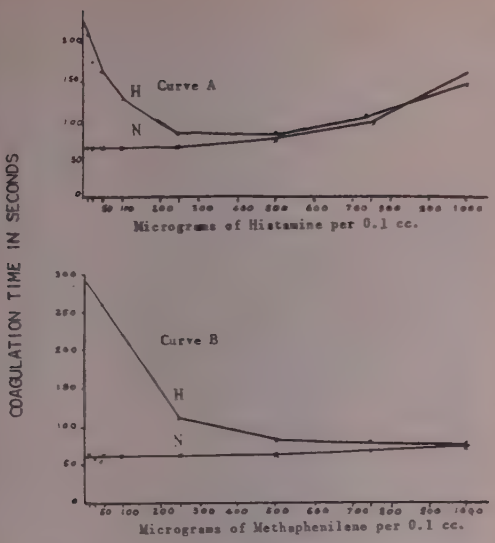

FIG. 1.

Effect of drugs on normal and heparinized rabbit plasma.

H = heparinized plasma
N = Normal plasma

Curve A—Histamine, Phenergan, Tagathen, Ambodryl, Anthallan, chlorocyclizine, Foralamin, Chloroprophenpyridamine, Doxylamine, phenindamine, C-5581-H.

Curve B—Methaphenilene, pyranisamine, tripelennamine, phenazoline, diphenhydramine, PD-AH-853, 194-B, p-fluorobenzyl D.P.E., Pyrathiazine No. 204.

obtained by us using rabbit plasma. However, the concentrations of added thromboplastin and platelets in our experiments were the same in the normal and the heparinized plasma so that any increase in thromboplastin due to platelet disintegration should be reflected in a decreased coagulation time in the normal plasma. Inasmuch as this did not occur, it is probable that the mode of action of both histamine and the antihistaminics as heparin inactivators is by direct chemical combination similar to that observed with the dyes(3,4).

Summary. The effects of histamine and a large number of antihistaminic drugs upon the coagulation of normal and heparinized rabbit plasma have been investigated. It has been established that the amount of each compound required for an antiheparin effect is critical. At concentrations below the critical level the drugs have little or no effect upon the coagulation system studied. At concentrations above this level several of the antihistaminics as well as histamine produced a progressively increased incoagulability of the system. This anticoagulant effect is related to concentration and the action is on some substance other than heparin. Thenfadil, methapyrilene and thonzylamine had no effect upon normal or heparinized plasma. Of all the compounds tested, 194-B, methaphenilene, pyranisamine, 204, phenazoline, tripelennamine, diphenhydramine, pyrathiazine, P.D. AH-853 and p-Fluorobenzyl D.P.E. gave the best results in that, upon reaching the critical concentration for heparin inactivation, they did not become anticoagulants themselves. It has been postulated that the antiheparin activity of these compounds is due to their direct combination with heparin.

The authors wish to thank the Upjohn Co. for the heparin and the Pyrolozote; Parke-Davis & Co. for the Benadryl, AH-853 and Ambodryl; Ciba Pharmaceutical Products, for the Pyribenzamine, 204, and Antistine; Merck & Co. for the Neo-Antergan and Phenergan; Nepera Chemical Corp. for the Neo-Hetramine; Lederle Laboratories for the p-Fluorobenzyl D.P.E. and the Tagathen; Wm. R. Warner for the Diatrin; Wm. S. Merrell for the Decapryn; Abbott Laboratories for the Thenylene and Di-Paralene; Winthrop-Stearns for the Thenfadil; Schering Corp. for the Chloro-Trimeton; Burroughs-Wellcome for the Perazil; Hoffmann-La Roche for the Thephorin; Medico Chemical Specialties Corp. for the Anthallan; White Laboratories for the 194-B; Bristol Laboratories for the C-5581H; and Eaton Laboratories, Inc., for Foralamin.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Experimental Deflection of the Course of Optic Nerve in the Chick Embryo.* (18471)

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The fibers of the optic nerve of vertebrates grow from the ganglion cells of the retina into the diencephalon, where the bundles from both sides intersect to form the optic chiasma. The present study is a first attempt to analyze the factors determining their orientation and distribution, particularly the guiding role of preformed pathways and the selectivity of associations (see Weiss)(1). In normal development, the epithelial tube of the optic stalk serves as guide channel for fiber growth between eye and brain. It was of interest, therefore, to investigate the course the fibers would take if deprived of this channel.

Materials and methods. Since the first optic fibers in the chick embryo reach the future chiasma region after 70 hours of incubation (2,3), the stalk had to be severed and its stump deflected at approximately this stage. Into an incision between the eyes made by a fine glass needle, a triangular piece of shell membrane, cca 0.2 mm long, was introduced as a barrier. These fragments had previously been immersed in glycerine-egg albumen, then in alcohol for coagulation, then stained with Nile blue sulphate and finally washed in boiled water. The eggs were closed again with caps of egg shell(4). Four to 5 days p. op. the embryos were fixed, stained and sectioned (Bodian's formalin-protargol; Cajal's pyridine-silver).

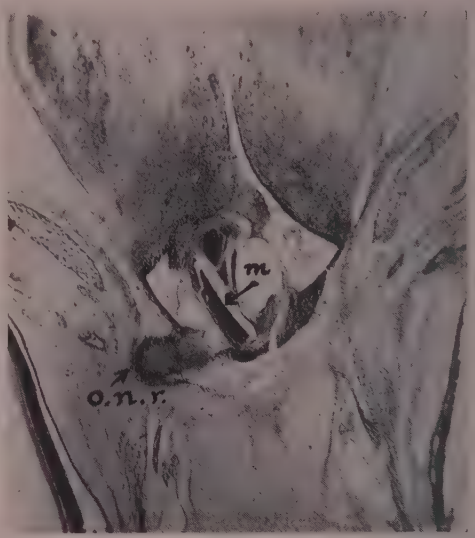

Fig. 1.

Fig. 1 and 2. Sections through chiasma region at different levels of same chick embryo with shell membrane (m) blocking and deflecting right optic nerve (o.n.r.); course of left optic nerve (o.n.l.) normal. × 22.

Results. In most of the 30 otherwise successful cases, the membrane was found displaced at some distance from the chiasma. In these cases, the optic nerves and chiasmata were normal, indicating that the severed stumps of the stalk had healed and restored a normal growth channel. In 6 cases, however, the membrane had remained in the desired blocking position and deflected the outgrowing nerve fibers. In a typical case, shown at two different levels (Fig. 1 and 2), the membrane (m) can be seen in the path of the nerve (o.n.r., Fig. 1), whose course is then turned downward toward the pharynx (Fig. 2), in contrast to the opposite normal optic nerve (o.n.l.). Specifically, the results in the other 5 cases were as follows. *Cases B and C*: One optic nerve totally deflected toward the pharynx, the other divided into a diencephalic and a pharyngeal branch. *Case D*:

* Research done by the author as Rockefeller Fellow under the direction of Dr. Paul Weiss and aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and the United States Public Health Service.

1. Weiss, Paul, *Genetic Neurology,* University of Chicago Press, 1950.

2. Tello, J. F., *Trav. Lab. Rech. Biol. Univ. Madrid,* 1923, v21, 1.

3. Windle, W. F., and Austin, M. F., *J. Comp. Neur.,* 1936, v63, 431.

4. Price, J. W., and Fowler, E. V., *Science,* 1940, v91, 271.

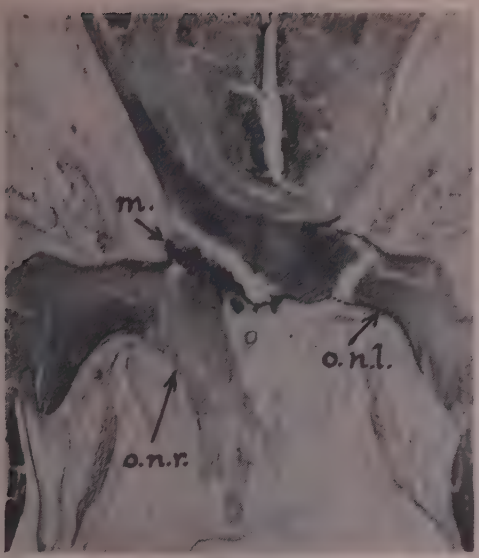

Fig. 2.

Both optic nerves blocked from meeting and from crossing midline, so that each terminates on its side of the brain, with part of one also deflected toward the pharynx. *Case E*: One nerve in direction of brain, but without entering it, terminates in connective tissue; the other with branches into both brain and pharynx. *Case F*: Both nerves deflected into pharynx.

After obstruction of their normal pathway the outgrowing fibers followed the matrix of the surrounding connective tissue which led them to abnormal destinations. Knoblike endings of these optic stray fibers were seen in the connective tissue, in perichondrium and in the pharyngeal epithelium. Even in their abnormal courses, the outgrowing fibers tended to associate into solid bundles by fasciculation(5). Yet there was no evidence of any neurotropic attraction toward the brain. On the other hand, once the fibers had entered the brain wall, their courses and terminations seemed to tend to become typical. The results are in agreement with the principles of "contact guidance"(1) and "selective fasciculation(1)."

Incidentally, cases with only a single optic nerve penetrating the brain furnished an experimental demonstration of the partial decussation of optic fibers in the chick (Fig. 3).

Summary. The effects of early transection and mechanical obstruction of the optic nerve in the chick embryo were studied. The deflected fibers follow abnormal courses determined by the surrounding connective tissue matrix without evidence of neurotropic attraction by the brain.

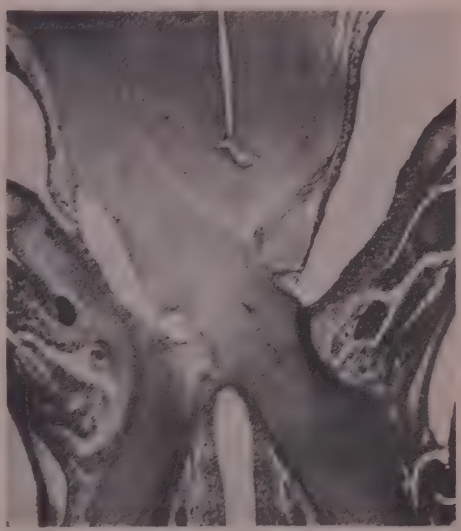

Fig. 3.
Chick embryo with deflected right optic nerve, showing partial decussation of fibers of unaffected left optic nerve. × 27.

5. Weiss, Paul, *J. Exp. Zool.*, 1950, v113, 397.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Inverse Changes of Serum Glucuronidase and Esterase of Breast Cancer Patients on Estrogen Therapy.* (18472)

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It was pointed out(1) that during estrogen therapy of patients with advanced breast cancer the levels of serum β-glucuronidase were markedly elevated. Harris and Cohen (2) in studies on the effects of ovarian hormones on tissue enzyme activities in mice observed that in most instances there appeared to be an inverse relationship in the tissue glucuronidase and esterase activity responses. Thus, for example, while the uterine glucuronidase level was reduced to about one-half by ovariectomy, the esterase activity was almost doubled; both changes were reversed by the administration of estrogen. It was thought possible that a similar relationship of glucuronidase and esterase changes in the blood of patients with breast cancer and on sex hormone therapy might occur. The glucuronidase and esterase activities were therefore simultaneously determined for the sera of patients during various stages of sex hormone therapy. The data obtained definitely indicate an inverse relationship between the glucuronidase and esterase changes in these patients.

Methods. The selection and treatment of the patients followed the same criteria as that previously reported(1). Serum glucuronidase was determined by the method of Fishman *et al.*(3), using the modified order of reagent additions(4). Serum esterase was determined titrimetrically using a procedure modified from the histochemical procedure of Glick(5). The sera were first diluted 3:10 with distilled water and all reagents were used in amounts about 150 times as great as those proposed by Glick in order that the test be applicable on a macro scale. One enzyme unit is defined as that amount of enzyme which liberates 1 γ of phenolthalein or butyric acid per hour incubation from their corresponding substrates by the glucuronidase and esterase respectively.

Results. A total of 37 healthy control subjects,† 11 patients with advanced breast cancer and not on hormone therapy, and 12 patients with advanced breast cancer on estrogen therapy‡ (3 patients were being treated with ethinyl estradiol and 9 with diethyl stilbestrol) were studied in this series of experiments.

Fig. 1 shows values obtained for serum glucuronidase and the corresponding esterase activity levels obtained for (a) a group of healthy male subjects (ages 48-61, average 53), (b) a group of healthy female subjects (ages 47-69, average 53), (c) a group of patients (ages 45-71, average 62) with advanced breast cancer who were not on any hormone therapy, (d) patients (ages 52-71, average 62) on estrogen therapy whose glucuronidase levels were less than 1500 units %, and for (e) patients (ages 42-73, average 62) on estrogen therapy who showed glucuronidase levels greater than 1500 units %

* This work was aided by grants from the American Cancer Society, through an Institutional grant made to the University of Minnesota, and the University of Minnesota Graduate School.

1. Cohen, S. L., and Huseby, R. A., *Cancer Research*, 1950, v11, 52.

2. Harris, R. S., and Cohen, S. L., *Endocrinology*, in press.

3. Fishman, W. H., Springer, B., and Brunetti, R., *J. Biol. Chem.*, 1948, v173, 449.

4. Fishman, W. H., Kasdon, S. C., and Homburger, F., *J. Am. Med. Assn.*, 1950, v143, 350.

5. Glick, D., *Z. Physiol. Chem.*, 1934, v223, 252.

† Sera was obtained from these control subjects through the Cancer Detection Center of the University of Minnesota Hospitals.

‡ The hormones used in these studies were supplied through the Committee on Research of the Council on Pharmacy and Chemistry of the American Medical Association by the Abbott Research Laboratories, Schering Corporation, and Winthrop-Stearns.

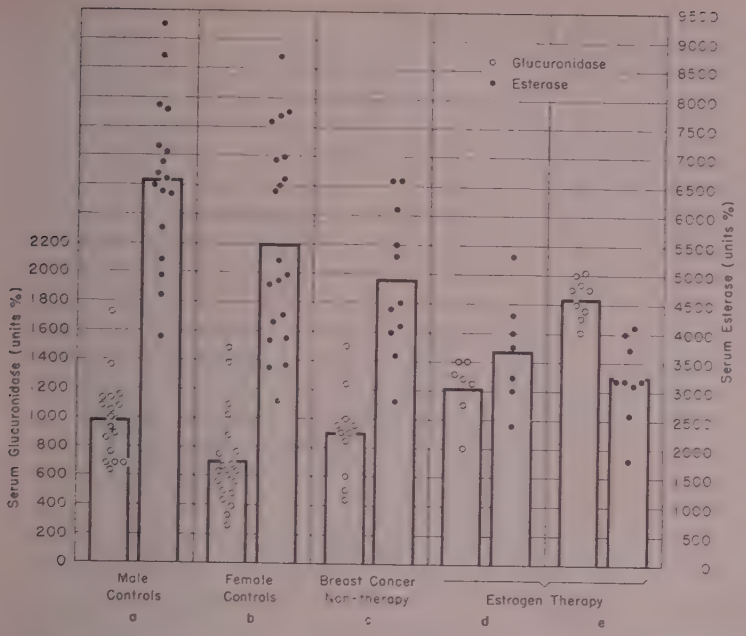

FIG. 1.
Glucuronidase and esterase values in normal and breast cancer patients. For description see text.

(several patients contributed values used in more than one category of (c), (d) and (e)—*e.g.* patient A.M., Fig. 2b). Where several assays were available for a single patient in any one of these categories, these were averaged and each point in the various categories thus represents a different subject.

A comparison of the post-age 50 healthy male and female subjects shows: 1. A significantly higher average serum glucuronidase in males (990 ± 40)§ than in females (700 ± 65). This observation is in agreement with the findings reported earlier(1).

2. The serum esterase levels in the males (6600 ± 300) is likewise significantly higher than in the females (5500 ± 400) of this group.

The group of non-treated breast cancer patients showed a just significant increased serum glucuronidase activity level (900 ± 95) as compared to that of the healthy women. The non-treated cancerous patients showed on the other hand a lower (4800 ± 600) serum esterase than the healthy controls. The patients on estrogen therapy showed correspondingly lower esterase values (3700 ± 400) corresponding to glucuronidase values below 1500 units % (average 1220 ± 160) and still lower (3260 ± 250) for those glucuronidase values above 1500 (average 1840 ± 75).

The inverse relationship between serum glucuronidase and esterase levels is even more clearly demonstrated by following the changing enzyme activity concentrations in individual patients during and following endocrine therapy. In Fig. 2 are shown the enzyme activity concentrations for (a) a patient (E. F.) on diethyl stilbestrol therapy (15 mg/day); (b) a patient (A.M.) on ethyinl estradiol therapy (3 mg/day); (c) a patient (E.B.) from whom stilbestrol was withdrawn after 550 days of treatment; and for (d) a patient (E.H.) from whom estradiol was withdrawn after 275 days of treatment. It will be seen that in all cases there tends to be an inverse relationship between changing

§ The $\pm$ refers to the standard error.

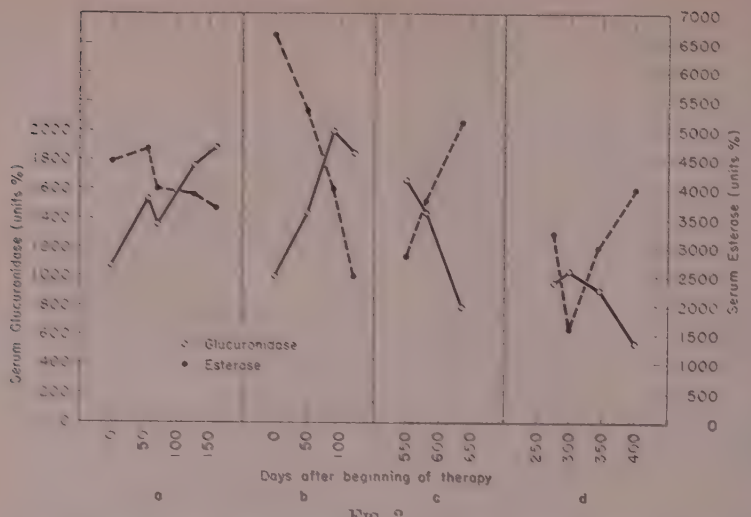

FIG. 2.

Glucuronidase and esterase changes during and following estrogen therapy to breast cancer patients. For description see text.

serum glucuronidase and serum esterase. Similar observations have been made on all the other patients studied.

Discussion. The data presented in this paper clearly illustrate that with a rising serum β-glucuronidase there is a reduced serum esterase and that with a falling glucuronidase there is an increased serum esterase. That these phenomena are not limited to cancer patients being treated with estrogens is attested to by the following observations: 1. Similar changes were frequently observed for various tissues in mice subjected to changes in the amount of circulating ovarian hormones(2). 2. Preliminary observations on mammary tumors in mice(6) indicate reduced esterase levels accompanying the increased glucuronidase activities levels in the tumorous tissue. 3. In a few scattered observations(7) where definite changes in serum glucuronidase levels have been found in subjects for whom several enzyme determinations have been made on different occasions, inverse changes in serum esterase levels have been observed.

More frequent determinations will have to be made in order to determine whether the changes in glucuronidase and esterase levels occur simultaneously or whether one change precedes the other. The data so far obtained, while far from conclusive, also tend to indicate that the relative degree of esterase and glucuronidase changes varies for different patients, and even in the same patient does not appear to be constant from time to time.

In view of the almost complete lack of evidence or hypotheses with regard to the function of esterase in the body and the suggested, though far from proven, hypotheses for glucuronidase function(2,8,9), it is felt that no interpretation can be made for the data presented in this paper. Two possibilities which have suggested themselves is that the esterase serves as a precursor for the more specific enzyme glucuronidase, or that some tissue or tissues involved in the production of esterase may under certain circumstances preferentially produce the more specific enzymes. However, until some evidence is available with regard to these suggestions they must be considered as interesting though highly speculative hypotheses.

Summary. Simultaneous sera glucuronidase and esterase determinations have been carried

6. Cohen, S. L., and Bittner, J., unpublished.
7. Cohen, S. L., unpublished.
8. Fishman, W. H., *J. Biol. Chem.*, 1947, v169, 7.
9. Kerr, L. M. H., Levvy, G. A., and Campbell, J. G., *Nature*, 1947, v160, 572.

out on the blood samples of 37 post-age 50 healthy control subjects (17 males and 20 females), 11 patients with advanced breast cancer and not on hormone therapy, and on 12 patients with advanced breast cancer receiving estrogen therapy. It was found that the control male subjects had both a higher serum glucuronidase and serum esterase than did the control female subjects. An inverse relationship was found for the changes in sera activity levels of glucuronidase and esterase in the patients with breast cancer subjected to variations in estrogen therapy.

We wish to thank Ruth Slaton Harris and Shirley Chapman Tennyson for their technical assistance in carrying out the assays reported in this paper.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Synnematin, an Antibiotic Produced by *Tilachlidium*. 18473

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(Introduced by C. W. Muehlberger)

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In a search for microorganisms capable of producing antibiotic substances, a mold belonging to the genus *Tilachlidium* Preuss which inhibits bacterial growth was isolated. In liquid medium the mold forms a substance which is more active against certain species of *Salmonella* than against *Escherichia coli* and which has low toxicity for mice. On the basis of the characteristic hyphal fascicles (Synnemata) of *Tilachlidium*, the name "synnematin" is proposed for the antibiotic.

Materials and methods. (a) *Method of assay.* One synnematin unit is defined as the minimum quantity/ml which completely inhibits the growth of *S. typhimurium* for 24 hours. Activity is assayed by the broth dilution test with *S. typhimurium* (Dr. P. R. Edwards, strain 9) as the test organism. Samples for assay are sterilized by Seitz filtration. .F. D. A. broth* at pH 7.0 is inoculated with 1% of an 18 to 24 hour culture and 2-fold dilutions of the antibiotic are made with 2.0 ml volumes of the seeded broth. (b) *Production.* The synnematin used in these studies was produced by growing *Tilachlidium* sp. strain MDH 3590A, on a medium composed of Bacto Casamino Acids, 10.0 g; glucose, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; and $FeSO_4 \cdot 7H_2O$, 0.1 g/liter of tap water. The medium was adjusted to pH 6.7-6.9, dispensed in 2-quart milk bottles in 300 ml volumes and autoclaved at 121°C for 30 minutes. Each bottle was seeded with 6 ml of a 4-day-old culture in Czapek-Dox medium and incubated on the flat side at 24°C. Peak activity, 8 to 32 units, was obtained in 6 to 9 days. At this time the pH was 7.9 to 8.3. (c) *Purification.* The culture liquid was adjusted to pH 6 with H_3PO_4, mixed with 0.25% Nuchar C-190-N and filtered with the aid of 0.5% Standard Super-Cel. The antibiotic was then adsorbed from the filtrate on 1% Darco G-60. Synnematin was eluted from the Darco with 0.1 volume of 75% acetone, concentrated in vacuum at a temperature under 30°C and the residue was clarified with 2% Nuchar. The resulting filtrate was shell-frozen and dried. Crystalline synnematin has not been isolated: the most pure material thus far obtained contains 32 units/mg.

Characteristics. (a) *Stability.* Synnematin

TABLE I. Effect of pH on Activity of Synnematin.

	Units/ml for inhibition	
Broth, pH	*S. typhimurium*	*M. pyogenes*
5.8	1	2
7.0	1	4
7.9	2	8

* Unless otherwise stated, "broth" or F. D. A. broth refers to the nutrient broth portion of penicillin assay agar(1).

1. Federal Register, 1945, 10F.R., 11478.

TABLE II. Activity of Synnematin Against Bacteria and Molds.

Organism	Strain No.	Test medium	Units/ml required for inhibition
Aerobacter aerogenes	527	FDA broth	>64
" "	600	" "	>64
" "	8308	" "	64
" "	8329	" "	>64
" "	618	" "	>64
Bacillus subtilis	231	" "	8
Brucella abortus	819a	Tryptose broth	2
" *melitensis*	812	" "	1
" *suis*	811	" "	1
Clostridium perfringens	BP6K	Mueller's seed medium	16
Corynebacterium diphtheriae	PW8	Veal infusion broth	0.25
Diplococcus pneumoniae Type III		Felton's medium	1
Escherichia coli	8090	FDA broth	>64
" " var. *communior*	603	" "	32
" " var. "	621	" "	32
" " var. *communis*	605	" "	64
Hemophilus pertussis		B-G medium	16
Klebsiella pneumoniae		FDA broth	>32
Micrococcus pyogenes var. *aureus*	SM	" "	1
" " var. "	209P	" "	2
" " var. "	B314	" "	2
Mycobacterium tuberculosis var. *hominis*	H37	Glycerol beef extract agar	>12
" *phlei*	1201	" " " "	>12
Proteus sp.	1414	FDA broth	1
" *morganii*	548	" "	>64
Pseudomonas aeruginosa	R-P-CC	" "	>32
Salmonella cholerasuis	550	" "	1
" *enteritidis*	575	" "	1
" *paratyphi*	577	" "	1
" *pullorum*	75	" "	0.5
" *schottmülleri*	576	" "	1
" *typhimurium*	9	" "	1
" "	569	" "	1
" "	SB682	" "	2
" "	SB683	" "	2
" *typhosa*	710	" "	0.5
" "	17,001	" "	0.5
Shigella alkalescens	801	" "	16
" "	802	" "	4
" "	1044	" "	8
" *dysenteriae* Flexner X	3001	" "	32
" *Lavington*		" "	1
" *madampensis* Dispar I		" "	64
" *paradysenteriae*	1006V	" "	64
" "	2003W	" "	64
" "	8031	" "	64
" "	9001	" "	64
" "	10,001	" "	64
" "	11,001	" "	64
" "	12,001	" "	64
" "	15,001	" "	4
" "	15,401	" "	32
" "	15,802	" "	8
" "	15,601	" "	64
" *rabaulensis*	16,002	" "	16
" *schmitzii*	202	" "	4
" *shiga* Shiga I		" "	32
" *sonnei*	401	" "	64
Streptococcus hemolyticus	98A	Felton's	1
" "	286	"	32
" "	314B	"	4
" "	316A	"	32
" "	594	"	2
" "	M1-1A	"	2

TABLE II (continued).

Streptococcus hemolyticus	SF IV	Felton's	1
" "	K64C	"	4
Aspergillus flavus	1035	FDA agar	>12
" *niger*	7416	" "	>12
Histoplasma capsulatum		" "	>12
Mucor sp.	8445	" "	>12
Penicillium notatum	832	" "	>12
Streptomyces griseus	4	" "	>12
Trichophyton mentagrophytes var. *gypseum*		" "	>12

is labile to acid and to heat. At pH 2, the activity disappears after 20 minutes at 70°C or 3 hours at room temperature but remains unchanged at 5°C for 45 minutes. Heating at 117°C for 10 minutes reduces the activity at pH 5 or 7.5 and destroys it at pH 9. Dried synnematin is stable for 11 months but shows a decrease in potency after 17 months at 5°C. (b) *Extractability.* Synnematin is not extracted from culture filtrates by amyl acetate, n-butanol, chloroform or diethyl ether at pH 2, 4, 6 or 8. (c) *Solubility.* It is readily soluble in water, methanol, 75% acetone or 50% ethanol and insoluble in ethanol, acetone and diethyl ether.

Effect of pH, addition of serum, and size of inoculum on activity. The data in Table I show that the antibiotic is more active in a slightly acid medium than in an alkaline one. In broth containing 20% horse serum, approximately twice as much synnematin is required to inhibit *S. typhimurium* and *M. pyogenes* as is needed in plain broth. The size of inoculum has little effect on the concentrations necessary to prevent growth. One unit inhibited concentrations of *S. typhimurium* whether the number of organisms used for inoculum was 7,000 or 70,000,000/ml.

Biological properties. (a) *Activity of Synnematin against bacteria and molds.* The antimicrobial spectrum of synnematin against various bacteria and molds is given in Table II. All of the tests were read in 24 hours except those for *Corynebacterium diphtheriae* which was read after 3 days incubation; *M. tuberculosis,* 42 days; *M. phlei,* 7 days; and the molds, 8 to 14 days. The inoculum was 1% of a 24-hour culture of the organisms in all the tests except for *C. diphtheriae* in which a loopful of pellicle from a 3-day culture was floated on the surface of the tubes of serially diluted synnematin and for *H. pertussis, M. tuberculosis, M. phlei* and the molds which were inoculated by streaking the surface of the agar slants containing dilutions of synnematin. (b) *Bactericidal activity of synnematin.* Table III shows that synnematin reduced the number of organisms but even 16 to 32 units does not sterilize the cultures. A series of tubes containing 2-fold dilutions of synnematin in broth was inoculated with an equal quantity of broth seeded with 1% of an 18 to 24 hour culture *M. pyogenes.* Another series of tubes was similarly inoculated with *S. typhimurium.* The number of organisms present before and after 24 hours incubation at 37°C was determined by plating on nutrient agar. In the test made with *M. pyogenes* there were more organisms present in the tube containing 16 or 32 units than in the tube containing only one unit of synnematin. Similar results were obtained in several other experiments conducted in the same manner. It is possible that this phenomenon is the same as the one described for

TABLE III. Reduction of No. of Organisms in Presence of Synnematin.

Test organism	Exp. No.	Units of antibiotic	No. of organisms before incubation, ×1000	No. of organisms after incubation.
M. pyogenes	1	1	540	90
		32	540	260000
	2	1	500	300
		16	500	100000
S. typhimurium	1	1	1300	80
		32	1300	270
	2	1	920	36
		32	920	20

TABLE IV. Resistance to Synnematin Induced in Three Organisms by Daily Transfer in Broth Containing the Antibiotic.

Transfer No.	Units of synnematin required for complete inhibition		
	S. typhimurium	*M. pyogenes*	*D. pneumoniae* Type III
1	1	4	1
2	4	4	2
3	8	8	2
4	8	8	2
5	8	8	4
6	8	8	4
7	16	16	4
8	16	16	4
9	16	16	8
10	32	16	8
11	32	32	8
12	32	32	8

TABLE V. Effect of Synnematin on Mice Infected with *D. pneumoniae* Type III. (6 mice in each series).

Dilution of culture	Dose of synnematin	No. survived
1:10000000	None, control	4
1:1000000	None, control	1
1:10000	800 units in 1 dose	6
1:100	800 units in 1 dose	3
1:100	1600 units in 2 doses, 8 hr apart	6

penicillin by Eagle(2). (c) *Induced resistance developed toward synnematin.* Serial 2-fold dilutions of synnematin containing 128 units/ml were made in broth seeded with *M. pyogenes* or *S. typhimurium* or, in Felton's broth seeded with *D. pneumoniae.* Twenty-four hours later a similar series of tubes was made in which the 1% inoculum was taken from the tube containing the highest concentrations of antibiotic permitting growth. These daily transfers were repeated 12 times. The results given in Table IV show that the resistance of *S. typhimurium* toward synnematin increased 32 fold, but in the case of *M. pyogenes* and *D. pneumoniae* the resistance increased only 8-fold. (d) *Toxicity.* When administered intraperitoneally, 100 mg of partially purified synnematin was tolerated by 20 g mice. Four hundred mg was not lethal when 100 mg of the material was injected in 2 equally divided doses daily over a period of 4 days. One hundred mg contained 3200 synnematin units. Injection of larger doses awaits further purification of the antibiotic. (e) *In vivo activity.* Preliminary experiments were made to determine whether synnematin protected mice after injection with *D. pneumoniae* Type III and chick embryos after injection with *S. pullorum.*

Table V shows the effect of the treatment of mice with synnematin after intraabdominal injection of 0.1 ml of dilutions of a 24-hour culture of *D. pneumoniae* Type III. The single dose, or the first dose when 2 doses were given, was administered subcutaneously immediately after the injection of the organisms. The animals were observed for 14 days.

Table VI shows the effect of the treatment of chick embryos after infection with 0.1 ml of dilutions of a 24-hour culture of *S. pullorum.* Eleven-day-old chick embryos were used and all injections were made into the allantoic cavity. The synnematin was given immediately after the injection of organisms. After further incubation for 8 days the eggs were cultured in both nutrient and tetrathionate broths. Cultures were plated on SS agar and on bismuth sulfite agar.

Discussion. The form-genus *Tilachlidium* includes those imperfect fungi having *Cephalosporium*-like conidial heads and conidiophores aggregated into synnemata. Waksman and Horning(3) included the *Cephalosporium-Fusarium* group among the fungi known to be capable of producing antibiotics, and there may be a phylogenetic relationship between the strains reported here and other antibiotic-forming molds. In fact, broth cultures of *Cephalosporium charticola* Lindau have

TABLE VI. Effect of Synnematin on Chick Embryos Infected with *S. pullorum.* (6 embryos in each series).

Dilution of culture	Dose of synnematin	No. of uninfected embryos
1:1000000000	None, control	5
1:100000000	None, control	1
1:10000000	None, control	0
1:100000	320 units in 2 doses, 24 hr apart	6

2. Eagle, H., *Science,* 1948, v107, 163.

3. Waksman, S. A., and Horning, E. S., *Mycologia,* 1943, v35, 47.

been found to contain synnematin(4). All the tests were made with partially purified synnematin and further purification may establish the presence of more than one chemical entity. The material investigated has certain attributes of a clinically desirable antibiotic such as: (1) It is soluble in water. (2) It is stable and active near neutrality. (3) It is active against a large number of species of bacteria. (4) The size of inoculum has little effect on the concentrations necessary to prevent growth. (5) Organisms do not appear to develop resistance rapidly. (6) It has a low toxicity for mice.

Summary. (a) A mold, a species of *Tilachlidium,* was found to produce a water soluble antibiotic, synnematin, which was partially purified. (b) Crude preparations inhibit *in vitro* certain species of *Brucella, Corynebacterium, Micrococcus, Streptococcus,* all species of *Salmonella* tested and some species of *Shigella.* Synnematin is inactive against the tested strains of *Aerobacter, Escherichia, Mycobacterium,* filamentous molds and most species of *Shigella.* (c) *In vivo* activity is demonstrated by the ability of synnematin to protect mice against infection with *D. pneumoniae* and to protect egg embryos against infection with *S. pullorum.* (d) Its low toxicity and other properties are reported.

4. Gottshall, R. Y., Roberts, J. M., Portwood, Lucile M., and Jennings, J. C., unpublished observations.

Received January 4, 1951. P.S.E.B.M., 1951, v76.

Influence of Storage on Viability of BGG Vaccine. (18474)

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The use of BCG vaccine has grown at a tremendous rate since the war despite the unsatisfactory nature of any prophylactic that consists of living and unstable microorganisms. The lack of a wholly acceptable preparation should not prevent such improvements as are possible within the formula originally established by Calmette(1) and, with this in mind, efforts to standardize BCG vaccine have been underway in the Division during the past five years. The present report of the uniformity of 204 consecutive weekly lots of vaccine and of their viability following storage is part of those studies.

Materials and methods. The methods have been described elsewhere(2). The last 144 lots of vaccine were processed from larger amounts of semidry BCG culture and were dispersed in a modified homogenizer making 60 r.p.m. In addition, the diluent has been replaced by a glycerine-free solution consisting of one part Sauton and three parts of phosphate buffer solution, pH 7.2. It is recognized that the homogenization of semidry BCG culture is incomplete. Direct examination shows small clumps of bacilli mixed with the more uniformly dispersed ones. The number of living BCG aggregates in each weekly lot of vaccine was determined by serial tenfold dilutions each seeded on five tubes of Löwenstein's egg medium. The colony counts were made after four and eight weeks' incubation at 37.5°C. The effect of storage was determined by vigorously shaking by hand various samples of vaccine that had been stored for as long as three and one-half years at 2-4°C. The viability tests were made of serial tenfold dilutions in Dubos' medium and on Löwenstein's egg medium.

Results. For purposes of simplicity, the results of the colony counts are shown in Table I as the mean of twelve consecutive weekly tests. As may be seen from the table,

1. Calmette, A., L'Infection bacillaire et la Tuberculose. Masson et Cie, Paris, 1936, p. 912.

2. Birkhaug, K., *Am. Rev. Tuberc.,* 1949, v59, 567.

the average for the entire period was 48 ± 10.55 colonies from 10^{-6} dilution of the 1 mg/ml BCG vaccine. A significant deviation from this mean occurred only during one period.

Viability of stored vaccine is shown in Table II. Approximately 50% of the BCG elements remained viable in the 3-week-old vaccine, 30% in the vaccine 6 weeks old, and 2% survived for one year.

TABLE I. Viability of BCG Vaccine Freshly Prepared from 10-11-Day-Old Sauton Culture.

Statistical analysis of number of colonies cultured on Löwenstein's egg medium from 10^{-6} suspension of 1 mg. ml BCG vaccine diluted in physiological saline solution (120 cultures in each group).

Weekly lots of BCG vaccine, wk	No. of colonies	Probability	
		t	*P*
1 to 12	45 ± 5.39*	—	—
13 to 24	44 ± 6.25	.162	.87
25 to 36	52 ± 11.49	1.715	.10
37 to 48	47 ± 7.49	.735	.47
49 to 60	44 ± 12.09	.245	.82
61 to 72	46 ± 9.39	.294	.77
73 to 84	38 ± 7.00	2.450	.03
85 to 96	47 ± 8.78	.612	.55
97 to 108	37 ± 6.33	2.695	.02
109 to 120	38 ± 6.32	2.694	.02
121 to 132	48 ± 8.71	.906	.38
133 to 144	43 ± 3.88	.808	.42
145 to 156	63 ± 24.40	2.205	.03
157 to 168	42 ± 8.25	.612	.55
169 to 180	61 ± 23.20	2.180	.04
181 to 192	68 ± 21.03	*5.550*	*<.01*
193 to 204	57 ± 9.17	2.709	.02

* Mean ± stand. dev. of the mean.
Significant deviation italicized.

TABLE II. Viability of BCG Vaccine Stored at 2-4°C Up to 3½ Years.

Vaccine Lot No.	Storage time	Colonies per ml of dilution of 1 mg/ml vaccine	% survival rate	Growth of BCG vaccine diluted in Dubos' medium (pos. a.f.b. smear)
A-90	Fresh	49×10^6	—	10^{-7}
"	1 day	45 "	92	10^{-7}
"	2 days	41 "	84	10^{-7}
"	3 "	46 "	92	10^{-7}
"	4 "	37 "	76	10^{-6}
"	5 "	41 "	84	10^{-7}
"	6 "	40 "	82	10^{-6}
"	7 "	38 "	78	10^{-6}
"	2 wk	26 "	53	10^{-5}
"	3 "	24 "	49	10^{-5}
"	4 "	19 "	39	10^{-5}
B-90	6 "	15 "	31	10^{-5}
A-87	3 mo.	9 "	18	10^{-5}
A-81	6 "	6 "	12	10^{-5}
A-75	9 "	12×10^5	2.5	10^{-4}
A-68	1 yr	9 "	1.8	10^{-4}
A-41	2 "	4×10^2	0.0008	10^{-1}
A-15	3 "	0	—	0
A-2	3½ "	0	—	0

Summary. Comparison of 204 consecutive weekly lots of BCG vaccine prepared under standardized conditions were found to be satisfactorily uniform in the number of viable organisms and clumps of organisms they contained. The survival rate of BCG vaccine stored at 2-4°C has been determined.

Received January 4, 1951. P.S.E.B.M., 1951, v76.

Oral Administration of Coxsackie Viruses to Newborn and Adult Mice.* (18475)

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Chimpanzees and cynomologus monkeys are readily infected by oral administration of Coxsackie or C virus, although with no apparent illness(1,2). In both species a virus carrier state is produced followed by the development of neutralizing antibodies to the infecting strain of virus. The present study is concerned with the susceptibility of newborn and adult mice, when tested for oral infection.

Virus strains. The following C virus strains were employed in the form of suspensions of

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Melnick, J. L., *Bull. N. Y. Acad. Med.*, 1950, v26, 342, and unpublished data.

2. Melnick, J. L., and Ledinko, N., *J. Immunol.*, 1949, v64, 101.

skinned, eviscerated carcasses of infected infant mice: Conn.-5, Texas-1, Easton-2, Easton-14, and High Point (Hi-Pt). They were isolated in this laboratory and their properties have been described(1,3). *Susceptibility of newborn mice.* Groups of 8 newborn mice were fed virus by means of a 0.25 ml syringe and a blunted 26 gauge needle which was inserted into the mouth, care being taken not to injure the buccal membrane. Each mouse was given 0.01 ml of virus but in many instances not all of the suspension was consumed. Mice were observed daily for a period of 2 weeks and those mice which survived at the end of this time were bled from the heart, the blood being used for neutralization tests. *Susceptibility of adult mice.* Small pieces of bread on petri plates were soaked with 1.5 ml of a 20 per cent suspension of Hi-Pt virus; these plates were then set in cages, each one containing 2 mice. This procedure was repeated on the following day and when all the virus had been consumed, the mice were reassembled into one cage. At the end of 2 weeks, the experiment was terminated and the animals bled from the heart.

Transmission of resistance to offspring. Fifteen adult female mice, 2 animals per cage, were fed 10% Hi-Pt virus as indicated above. When these mice had consumed the virus, they were distributed among 3 cages for mating, each cage housing 5 females and one male. The 3 colonies of mice were observed daily for the appearance of the young. Litters born to these mice were separated together with their mothers from the colony of mice and placed in individual cages. The newborn mice were then challenged by intraperitoneal inoculation of 10 to 100 LD_{50} doses of Hi-Pt virus within 48 hours of birth. Following inoculation they were nursed by their own mothers. Adult female mice were also inoculated with Hi-Pt virus by the intraperitoneal and subcutaneous routes, and their offspring also challenged according to the procedure already described(4). *Transmission of C virus from inoculated to uninoculated mice.* Two groups of 4 newborn mice (less than 24 hours old) were inoculated subcutaneously with Hi-Pt virus diluted 10^{-2}. Four uninoculated mice of the same age were placed in the box with each of the 2 inoculated groups. This was also done with Easton-2 and Easton-14 viruses, except that there were 8 mice in each group inoculated with 10^{-5} and 10^{-6} dilutions respectively. The mice were observed for 3 weeks following which they were bled for neutralization tests.

Neutralization tests. These tests were carried out as previously described(3), using 10-fold serial dilutions of serum and a constant amount of virus (100 ID_{50} doses). The serum-virus mixtures were incubated at room temperature for one hour and then inoculated intraperitoneally into newborn mice. The end-point was considered as being that serum dilution which conferred complete protection.

Results. Susceptibility of newborn mice. That newborn mice are susceptible to the oral administration of 5 different strains of C virus belonging to 4 different immunological types(4) is evident from the results given in Table I. Newborn mice contract the paralytic disease when fed virus diluted at least to 10^{-3} concentration of infected carcass. From the neutralization tests carried out with the blood of those mice surviving oral inoculation, it is apparent that not all of them became infected. The mice surviving oral administration of Conn.-5 virus in a concentration of 10^{-3} and 10^{-5}, showed neutralizing antibody titers of 1:100 and 1:10 respectively, whereas the blood of those mice surviving oral inoculation with 10^{-1} concentration did not respond with the development of antibodies. None of the survivors given Texas-1 virus had neutralizing antibodies in their blood. They were detected in two other instances (Easton-2 and Ohio-1). It is quite possible that mice which survived the apparent exposure to the more concentrated virus (10^{-1}) and which failed to develop antibodies may not have swallowed the virus given them.

Susceptibility of adult mice. As indicated in Table I, feeding Hi-Pt virus to adult

3. Melnick, J. L., and Ledinko, N., *J. Exp. Med.*, 1950, v95, 463.

4. Melnick, J. L., Clarke, N. A., and Kraft, L. M., *J. Exp. Med.*, 1950, v92, 449.

TABLE I. Response of Newborn (A) and Adult Mice (B) to Oral Administration of C Viruses.

Virus fed	Dilution	Fate of mice*	Neutral. antibody response of mice surviving after 2 wk. Dilution of serum giving complete protection
		A. Newborn mice	
Conn.-5	10^{-1}	8/13(7)	0
	10^{-3}	5/13(4)	1:100
	10^{-5}	1/12(0)	1:10
Texas-1	10^{-1}	12/17(8)	0
	10^{-3}	7/15(5)	0
	10^{-5}	0/16	0
Easton-2	10^{-1}	4/13(4)	1:10
	10^{-3}	1/16(1)	0
	10^{-5}	0/6	0
Ohio-1	10^{-1}	5/6(3)	nd
	10^{-3}	2/14(2)	1:10
	10^{-5}	1/16(1)	0
Hi-Pt	10^{-2}	6/9(5)	nd
	10^{-4}	2/8(0)	nd
	10^{-5}	0/4	nd
		B. Adult mice (lactating females)	
Hi-Pt	10^{-1}	0/25	0

nd = not done.
* The numerator indicates number of infected mice. The number in parentheses indicates the number of mice with observable paralysis.

TABLE II. Susceptibility of Mice Born of Mothers Fed or Inoculated Parenterally with High Point Virus.

Susceptibility of infant mice to 10-100 ID_{50} doses (mothers fed virus)	Susceptibility of infant mice to 100-1000 ID_{50} doses (mothers inoculated parenterally with virus)
34/39 (22)	8/74 (5)

lactating females did not induce signs of infection in these animals; the absence of infection is further indicated by their failure to develop neutralizing antibodies to this virus. The explanation offered above for the failure of newborn mice to develop antibodies after being fed virus does not obtain in this instance since all of the virus given the adults was definitely consumed. In addition to these animals we have observed many adult female mice who cannibalized their infected young without effect on the mothers. *Susceptibility of mice born of mothers fed or inoculated parenterally with virus.* Mice born of mothers fed virus were highly susceptible, since, as shown in Table II, 87 per cent of such mice succumbed to intraperitoneal inoculation with 10 to 100 ID_{50} doses of virus. This result is correlated with the fact that none of the mothers themselves became ill or developed antibodies. In contrast to the absence of resistance in the young born of mothers *fed* virus, those mice born of mothers *inoculated* parenterally with virus did possess a considerable degree of resistance to the challenge dose of virus. As indicated in Table II, about 90% of these newborn mice withstood the larger challenge dose.

Comparison of oral and subcutaneous inoculation. There is a striking difference in infectivity of C viruses depending upon the route of inoculation. The comparison given in Table III shows that the subcutaneous route of inoculation with each of the 5 C virus strains was about 10,000 times more effective in producing disease than the oral route. As may be seen, adult mice are not susceptible to either route of inoculation.

Transmission of C viruses from inoculated to uninoculated mice. The results in Table IV show that even direct contact between mice inoculated with virus and uninoculated

TABLE III. Comparison of Infectivity of C Viruses Administered Orally and Subcutaneously. (ID_{50}).

Virus	Subcutaneous	Oral
	A. Newborn mice	
Texas-1	$10^{-7.5}$	$10^{-3.1}$
Hi-Pt	$10^{-7.5}$	$10^{-3.5}$
Conn.-5	10^{-6}	$10^{-2.4}$
Ohio-1	$10^{-5.5}$	10^{-2}
Easton-2	10^{-5}	$10^{-0.7}$
	B. Adult mice (lactating females)	
Hi-Pt	0	0

TABLE IV. Fate of Uninoculated Mice in Direct Contact with Inoculated Mice.

Virus	Inoculated mice		Uninoculated contact mice		
	No. of mice	Incubation period (days)	No. of mice	No. with disease	No. with neutralizing antibodies
Hi-Pt	8	2	8	0	0
E-2	8	4–5	4	0	0
E-14	8	4–5	3	0	0

susceptible mice did not result in transmission of the disease. This is apparent by the absence of paralysis in the uninoculated mice and by the lack of development of neutralizing antibodies. In the course of work with these agents we have seen numerous examples in which uninoculated newborn mice have been kept with their inoculated littermates and only the latter developed disease.

Discussion. It is evident from the results obtained that young mice are susceptible to C viruses orally administered, whereas adult mice neither develop signs of disease nor produce antibodies following such exposure to virus. Von Magnus(5) has recently observed that newborn mice are also much more susceptible than adult mice to oral administration of the TO strain of mouse encephalomyelitis virus.

These results indicate that although one must consider the possibility of accidental contamination of newborn mice in the laboratory (in view of the finding of virus in the intestinal contents of infected mice), the concentrations of virus required to infect by the oral route are much higher than by other routes. This lessens the probability of accidental infections through this cause. Placing groups of uninoculated mice next to or even in with groups of infected mice has not resulted in infection of the uninoculated group.

The fact that some mice surviving oral infection possessed neutralizing antibodies in their blood and some did not deserves comment. The possibility exists that the latter mice were more resistant to the administered virus and that a variation in susceptibility exists among individuals of the same litter of newborn mice. On the other hand, the apparent resistances may be due merely to the technic employed and these animals may not have actually swallowed the virus. It appears more likely that with the more concentrated virus (10^{-1}), mice develop the apparent disease or are not at all infected.

Summary. (1) Newborn mice may be infected by oral administration of at least 5 different strains, representing 4 immunologically distinct Coxsackie, or C, viruses. Adult mice were resistant. (2) Newborn mice surviving oral administration may produce neutralizing antibodies. (3) Adult female mice fed C virus did not transmit neutralizing antibodies to their offspring, in contrast to females inoculated parenterally. (4) In a comparison of routes of inoculation of newborn mice it was found that higher titers may be reached by subcutaneous than by oral administration. The subcutaneous route proved to be about 10,000 times more sensitive than the oral route. (5) Direct contact between inoculated and uninoculated mice did not result in the transmission of infection.

5. von Magnus, H., *Acta Path. Microbiol. Scand.*, 1950, v27, 611.

Received January 8, 1951. P.S.E.B.M., 1951, v76.

Implanted Electrodes for Stimulating or Recording from Deep-Lying Brain Structures.* (18476)

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Recent studies (1-5) of brain stem mechanisms and their influences upon the electrical activity of the cortex have suggested the desirability of recording cortical and dience-

* Aided by a grant from the Commonwealth Fund.

1. Lindsley, D. B., Bowden, J. W., and Magoun, H. W., *EEG Clin. Neurophysiol.*, 1949, v1, 475.

2. Lindsley, D. B., Schreiner, L. H., Knowles, W. B., and Magoun, H. W., *EEG Clin. Neurophysiol.*, 1950, v2, 483.

3. Moruzzi, G., and Magoun, H. W., *EEG Clin. Neurophysiol.*, 1949, v1, 455.

4. Morison, R. S., Finley, K. H., and Lothrop, G. M., *Am. J. Physiol.*, 1943, v139, 410.

5. Starzl, T. E., and Magoun, H. W., *J. Neurophysiol.*, 1951, in press.

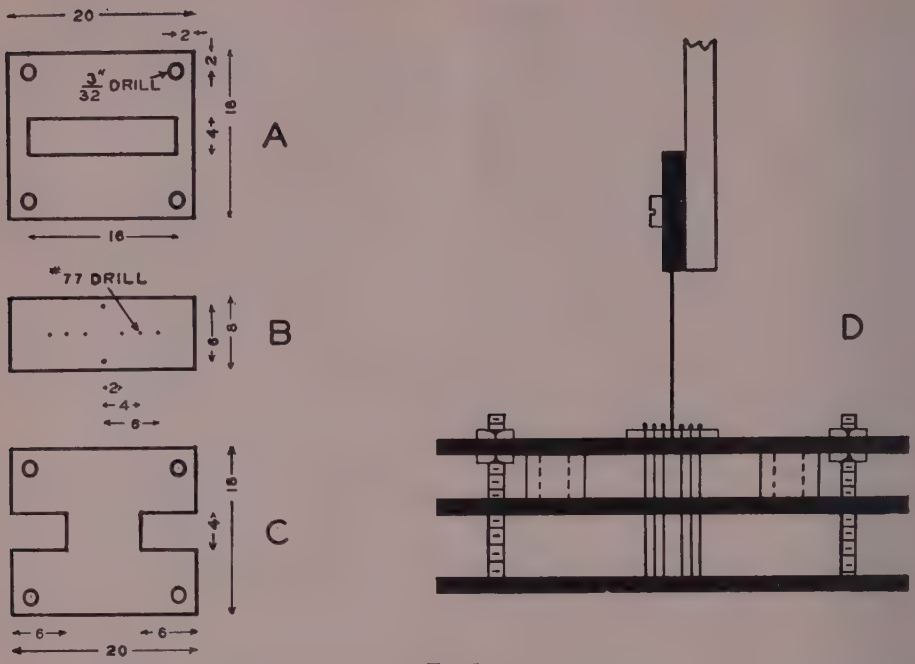

FIG. 1.

A. Standard base plate. *B*. Electrode carrier plate. *C*. Modified base plate used in reaching more lateral structures. *D*. Schematic representation of assembly jig and method of connecting electrode assembly to the Horsley-Clarke multiple electrode carrier. Dimensions are in millimeters.

phalic electrical activity in normal, unanesthetized cats during states of sleep and wakefulness. In order to record from or stimulate deep structures of the brain, a method of permanently implanting electrodes has been developed along the lines begun by Harris(6) and Hunter and Jasper(7). The possibility of wider application of this technic leads to detailed description here.

A permanently implanted electrode system for this purpose must meet three major requirements. It must be capable of *precise placement* or localization in order to explore systematically the matrix of small nuclei in the subcortical regions of the brain. It must be sufficiently *durable* to maintain electrical insulation in the presence of tissue fluids. It should not produce undue *tissue reactions* and irritability. A simple extension of the principles of stereotaxy permits accuracy of placement and satisfies the first criterion. Durability and non-irritability have been achieved by the proper selection of materials. Previous methods of implanting electrodes have used the Horsley-Clarke instrument for orienting the electrodes which are fixed into position by cementing them to sockets screwed into the calvarium. In the present method a new horizontal reference plane is established by attaching a flat base plate to the cranium parallel to the original Horsley-Clarke H (horizontal) reference plane. A second plate carrying the electrodes at predetermined H and L-R (left-right) positions is brought into place with the Horsley-Clarke instrument and cemented permanently to the base plate. In this manner several electrodes may be placed accurately to varying depths over a wide area.

Materials and procedures. The following sections describe in detail the construction of the various parts and sub-assemblies in this order. (1) The base plate. (2) The elec-

6. Harris, G. W., *Phil. Trans. Roy. Soc. Lond.*, 1946-47, v232 B, 385.

7. Hunter, J., and Jasper, H. H., *EEG Clin. Neurophysiol.*, 1949, v1, 305.

trode carrier plate. (3) The electrodes. (4) The assembly jig. (5) The electrode carrier assembly. After these discussions, the actual implanting operation is outlined and sample recordings are presented.

(1) *The base plate.* A base plate large enough to minimize leveling errors and yet small enough to be tolerated under the scalp is desired. Satisfactory dimensions are 18 mm in the A-P (anterior-posterior) dimension by 20 mm in the L-R dimension. Clear Plexiglas sheet 1/16″ thick is used because of its rigidity, lightness, and non-irritability. A hole, 4 mm A-P by 16 mm L-R, is cut in the center of the base plate to allow for the passage of the electrodes at the time of implantation. Holes (3/32″) drilled in the corners 2 mm from the edges accommodate the 2/56, 1/4″, stainless steel filister head screws which attach the plate to the skull (Fig. 1-A). With such a base plate six or eight electrodes may be implanted to any depth within the region bounded approximately by the A 15, P 2, and L-R 6 planes. To reach more laterally to L-R 10 or 12 a modified design is used (Fig. 1-C).

(2) *The electrode carrier plate.* A piece of 1/16″ Plexiglas sheet, 8 mm A-P by 20 mm L-R is next cut to form the electrode carrier plate. Center lines are inscribed on this plate and six No. 77 holes for the electrodes are drilled 2 mm apart at the desired L-R distances (Fig. 1-B). With the diameter of electrodes used, a 2 mm separation is necessary to prevent undue damage to the brain. Two No. 75 holes are drilled 6 mm apart on the midline to hold the rods by which the electrode assembly is attached to the Horsley-Clarke multiple electrode carrier. These connecting rods are made by crimping a 24-gauge stainless steel tube inside a 20-gauge tube leaving 1 mm of the 24-gauge tubing free to be cemented into the proper holes in the carrier plate.

(3) *The electrodes.* Several 6″ lengths of 26-gauge Nichrome wire are first drawn straight. The larger pieces are cut into a number of 40 mm electrodes and insulated with 5 coats of Belden Insulating Varnish. Each coat is baked at 400°F for 30 minutes. After the electrodes have been properly insulated, 3″ lengths of Grass insulated EEG electrode wire are soldered on for lead-off wires. The soldered joint is insulated with two coats of Tygon primer and one coat of Tygon plastic paint. After the insulation is checked, the electrodes are cut to the proper length and the tips sharpened and polished with emery cloth. It has been determined empirically that the *top of the base plate* will, on the average, lie in the H plus 24 plane. Variation from this mean value is rarely more than plus or minus one millimeter, so that in probing for large structures the error is not significant. However, if extreme accuracy is required, it is necessary to construct several electrode assemblies with electrodes of different lengths; then the appropriate one may be selected when the exact level of the base plate has been determined at the time of operation.

(4) *The assembly jig.* To facilitate the building of the electrode assembly, a small jig was constructed (Fig. 1-D). This jig consists of a floor plate and two adjustable guide plates of 1/8″ brass sheet, 3″ square. Two rows of No. 60 holes spaced 2 mm apart were drilled in both guide plates. One row is for the odd L-R measurement, *i.e.*, L-R 1, 3, 5, etc., and the other for the even distances. The guide plates are held together by 3 pins, while spacer sleeves maintain a separation of 10 mm between the plates. Three 6/32, 2″ bolts attach the floor plate to the guide plates and allow for adjusting the jig to electrodes of various lengths.

(5) *The electrode carrier assembly.* After the jig has been set to the proper H value, the electrode carrier plate is aligned with the proper holes in the guide plates and the electrodes are inserted and cemented in place with Testor's Household Cement. The ends of the connecting rods are dipped into cement and inserted into their holes in the carrier plate. The whole assembly is left in the jig for twenty-four hours until the cement has set; then the electrode carrier assembly is removed and the spacing and insulation of the electrodes is checked. Slight adjustments in alignment may be made by slipping

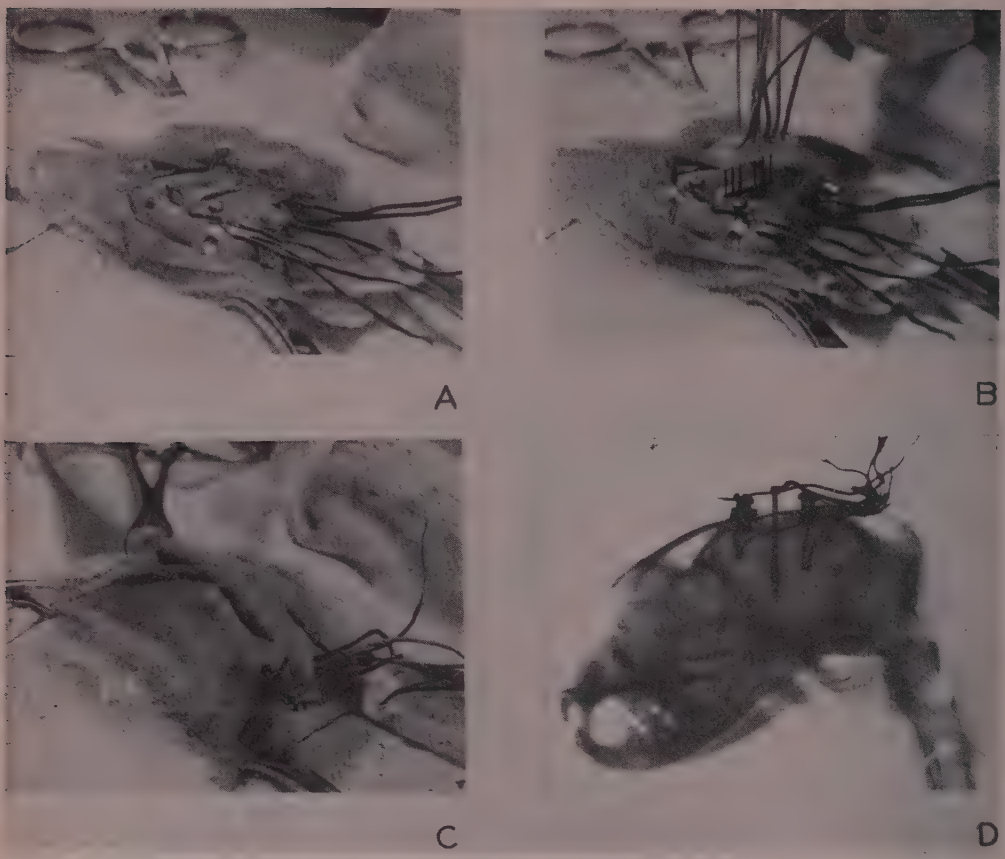

FIG. 2.

Progressive stages of the implanting operation. *A*. Placement of base plate. *B*. Insertion of electrodes. At this point ethylene dichloride is applied around the hole in the base plate and electrode assembly is lowered into place. *C*. Closure of incision over entire assembly with only lead-off wires protruding through stab wounds in skin at back of head. *D*. Lateral X-ray two weeks post-operative, showing six subcortical electrodes (straight vertical lines) reaching deep into the brain and six cortical electrodes (dark "bulbs" and wavy lines) resting on the dura. In all figures the animal is facing upper left.

a 20-gauge tube over the electrodes and bending them back into position. Tygon primer and plastic paint are now applied to the underside of the carrier plate around the electrodes to seal off the holes.

Implanting the electrodes. The electrodes are implanted under aseptic conditions. Zephiran Chloride, 1/1,000 aqueous solution, is used to sterilize the parts of the assembly. Two electrode carriers are required. A straight measuring probe is mounted in one of the carriers and calibrated in the usual manner. The second multiple electrode carrier holds the electrode assembly by means of the two connecting rods, and is calibrated for the A-P and L-R zero points. After the animal has been anesthetized (Nembutal-30 mg/K), shaved, mounted in the Horsley-Clarke instrument and draped, a midline incision is made along the top of the head, and the skin, muscles and periosteum are reflected. A 4 mm A-P by 20 mm L-R craniotomy is marked off over the proper A-P level with the aid of the measuring probe. After the bone has been trephined and rongeured away, the L-R calibration is checked and corrected by reference to the mid-sagittal sinus. Next, the 4 holes for the screws are laid out with

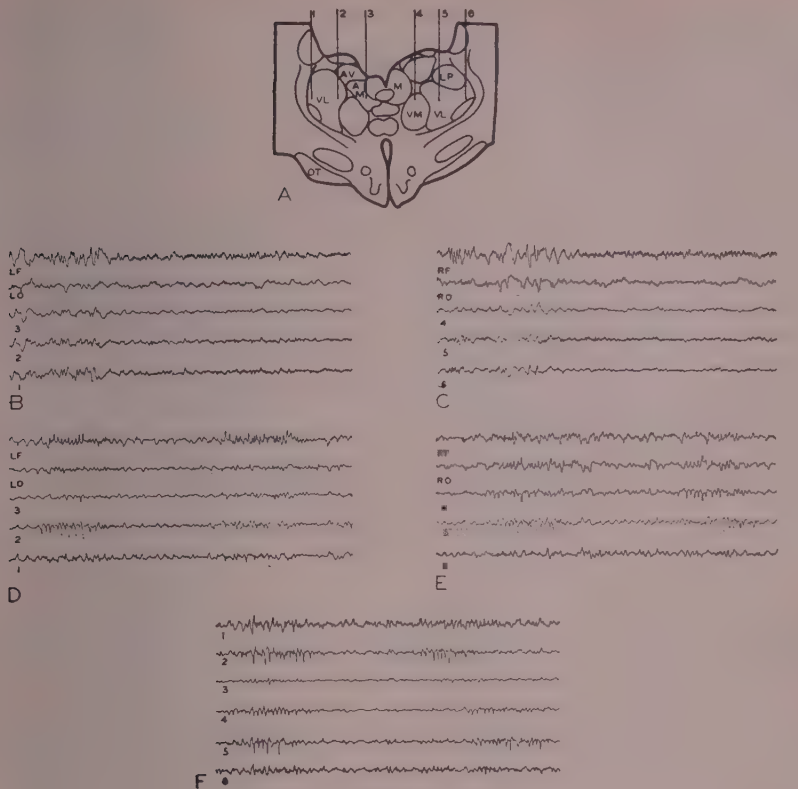

FIG. 3.

Section through rostral thalamus showing, *A*, placement of subcortical electrodes, and cortical and thalamic EEG's on the seventh post-operative day under conditions of normal spontaneous sleep and wakefulness, *B* and *C*; and under light barbiturate anesthesia (nembutal, 10 mg/k), *D*, *E*, and *F*. In records *B* and *C*, taken from the left and right sides of the brain, respectively, the animal is initially asleep and then wakens spontaneously. All tracings are monopolar to an "average" indifferent ground. The gain for the subcortical leads is 1.5 times that of the cortical records. The abbreviations are as follows: AV, anteroventral nuc.; AM, anteromedial nuc.; M, medial nuc.; VM, ventromedial nuc.; VL, ventralis lateralis nuc.; LP, lateralis posterior nuc.; OT, optic tract; LF, left frontal lead; LO, left occipital lead; RF, right frontal lead; RO, right occipital lead.

the measuring probe, drilled and tapped. Then a small amount of bone is whittled away from both sides of the craniotomy near the midline to create a broader foundation for the base plate, which is then attached to the skull and leveled with four stainless steel screws (Fig. 2-A). Again the measuring probe is used to check the leveling and to determine the final base plane. At times it is necessary to support one edge of the base plate with dental cement when the curvature of the skull is greater than usual. After the base plate is in place, the dura is slit and the electrode assembly is racked down until the carrier plate is about ¼" above the base plate (Fig. 2-B). A small amount of ethylene dichloride, the solvent for Plexiglas, is applied to the top of the base plate around the center hole and the electrode assembly is lowered into place. Three to 5 minutes are allowed for the junction to set and the connecting rods are removed. The lead-off wires are laid back along the skull and taken out through two stab wounds in the skin at the back of the head (Fig. 2-C). Closure is made in layers, making sure that the muscles are approxi-

mated as closely as possible around the lead-off wires. A clean dressing is applied and the animal is allowed to recover. Healthy animals generally do not require special post-operative care and rarely do they attempt to scratch at the wires. The animals remain docile and tractable and no difficulty has been experienced in soldering the lead-off wires to the EEG apparatus. When the animals have just been fed and are confined in a small, dark, warm, sound-proofed box, they drop off to sleep readily and continuous recordings can be taken over a period of hours. Examination of the brain of an animal with electrodes implanted for five months showed only negligible gliosis immediately around the electrodes and a slight herniation of the cortex through the slit in the dura. Accordingly, it is believed that by this method electrodes may be implanted in a normal brain and remain permanently serviceable.

Sample observations. The records shown in Fig. 3 were obtained from a cat on the seventh post-operative day. The animal was prepared as described above, and in addition 4 insulated, ball-tipped silver wires were inserted into small trephine holes to form the 4 cortical electrodes. These records are presented to demonstrate the feasibility of the method; a full account of studies on subcortical activity in normal wakefulness and sleep will be published at a later date.

Acknowledgment is made to Drs. H. W. Magoun, R. S. Snider, and D. B. Lindsley for laboratory facilities and guidance.

Received January 8, 1951. P.S.E.B.M., 1951, v76.

Temporary Accumulation of Eosinophilic Leucocytes in Spleen of Mice Following Administration of Cortisone. (18477)

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Allen(1) reported the occurrence of conspicuous numbers of eosinophilic leucocytes in the spleen in cases of sudden death caused by trauma and coronary occlusion. It was also noted that in the spleens of individuals who survived several hours the number of eosinophiles were progressively less as the period of survival increased. The explanation of this phenomenon was not known at that time. It was speculated, however, that it might be due to either histamine or acetylcholine release. Thorn(2) later described a test to ascertain the efficiency of the adrenal cortex by the disappearance of eosinophilic leucocytes from the circulating blood upon adrenal cortical stimulation by ACTH or adrenaline, or by injection of cortisone. It now appears most likely that the phenomenon described by Allen can be explained best by adrenal cortical stimulation during periods of stress and it would appear, from the observations in this report, that the eosinophiles upon disappearing from the circulating blood are stored temporarily within the spleen. In order to test this hypothesis the following investigation was undertaken:

Materials and methods. 24 male Swiss albino mice weighing 25 g each were divided into 2 groups of 12. One group was injected subcutaneously with 2.5 mg of cortisone acetate (Cortone acetate, Merck) and the other group served as controls. Eight hours post injection the animals were sacrificed by sharp blows on the back of the necks. This resulted in instantaneous death. All animals were necropsied; the spleen and thymus glands were fixed in formalin and in Zenker's solutions, were prepared for histologic examina-

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2. Thorn, G. W., Forsham, P. H., Prunty, F. T., Garnet, M. D., and Hills, A. G., *J.A.M.A.*, 1948, v137, 1005.

TABLE I. Comparison of the Average Number of Eosinophilic Leucocytes in the Spleen Per Oil Immersion Field in the Cortisone Treated Mice That Were Sacrificed 8 Hours Post Injection and in the Untreated Controls.

Mouse No.	1	2	3	4	5	6	7	8	9	10	11	12	Avg
Cortisone treated mice	1.7	1.9	.2	3.0	1.7	2.3	1.5	.8	1.0	1.2	.8	1.5	1.47
Control mice	.2	.2	.3	.1	.05	.2	.1	.3	.1	.2	.05	.2	0.16

tion, and stained with H & E and Giemsa stains. The spleens and the thymus glands of both groups of animals were examined under oil immersion magnification ($\times$ 950) and the eosinophilic leucocytes in each field were counted. Forty such fields were counted in each spleen with the edge of each field represented by the capsule of the spleen. Although the eosinophiles were present throughout the spleen, there was a tendency for more to collect near the capsule than in the splenic pulp elsewhere. The results of these counts are given in Table I. In addition six other similar mice were treated with 2.5 mg of cortisone and two were sacrificed 2, 4 and 6 hours post injection. These were studied as above.

Results. Examination of Table I indicates that there is a significant difference in the number of eosinophiles in the two groups studied. On the average there were 9 times as many eosinophilic leucocytes in the spleens of the cortisone acetate treated animals as in the untreated controls. It is also interesting that in one instance (Mouse No. 3) the cortisone treated animal did not show any increase in splenic eosinophilic leucocytes. In this animal it was noted that the usual reduction in size of the spleen that follows cortisone treatment did not occur. In 2 mice circulating eosinophile counts and in 3 mice the smears of peripheral blood from the tail veins were studied for eosinophilic leucocytes prior to injection with cortisone acetate and prior to sacrifice. These confirmed the fact that a marked drop occurred in the circulating eosinophilic leucocytes at the end of 8 hours in the cortisone acetate treated mice. The other treated mice that were sacrificed 2, 4 and 6 hours post injection did not reveal any significant increase in splenic eosinophilic leucocytes. In these latter animals the only changes noted was the progressive reduction in the sizes of the spleens and the disintegration of the lymphocytes that is typical of the alarm reaction. The thymus glands did not reveal any eosinophilia.

If it is postulated that in these experiments all, or the majority, of the circulating eosinophilic leucocytes are temporarily stored in the spleen it is important to calculate whether the spleen is large enough to store all the eosinophiles and whether the number of eosinophiles as seen in each oil immersion field could account for the number that disappeared from the circulating blood.

Weight of mouse = 25 g. Blood vol. 1/10 of body weight, 2.5 g = 2.5 cc; normal W.B.C. count 8,000 per cu mm; eosinophiles = 2% = 160 per cu mm.

Eosinophiles in total blood vol. = 160,000 x 2.5 = 400,000. Dimension of single eosinophile = 12 μ diameter. 1 square mm (1000 μ square) 12 μ thick would accommodate 83 x 83 eosinophiles = 6,889. 1 cubic mm would accommodate 6,889 x 83 = 571,787 eosinophiles. Or more than the total number of circulating eosinophiles. Size of normal mouse spleen is 20 x 5 x 3 mm = 300 cu mm. If *all* the circulating eosinophiles were stored in the spleen, *each cubic mm* would contain 1/300th of the total number of eosinophiles or 400,000 divided by 300 = 1333.

Since a cubic millimeter will accommodate 571,787 cells the size of eosinophiles it is necessary to calculate what percentage of 571,787 is 1333 eosinophiles, which is 0.233%. In one oil immersion field of sections of mouse spleen there are about 900 cells of all types which are approximately the same size as the eosinophiles. One per cent of 900 cells is 9 cells and 0.233% is slightly more than 2 eosinophiles per oil immersion field.

The spleens of the cortisone treated mice sacrificed at the end of about 8 hours were found to be about half normal in size. If only

one-half of the circulating eosinophiles were stored in these spleens the number of eosinophiles per oil immersion field would remain the same. Thus on the basis of these calculations, although approximate, it appears reasonable that the number of eosinophilic leucocytes seen in the spleen could account for the number disappearing from the circulating blood. Further investigations are being conducted in order to determine the fate of the eosinophilic leucocytes following adrenal cortical stimulation in splenectomized animals.

Summary. In a group of Swiss albino mice injected with cortisone acetate it was found that 8 hours post injection the spleens contained a significant increase in the number of eosinophilic leucocytes. It is postulated that this may be the locale of the eosinophilic leucocytes during their disappearance from the circulating blood following adrenal cortical stimulation. The number of eosinophilic leucocytes found in the spleen could account for the number that disappeared from the circulating blood following a single injection of cortisone acetate.

Received January 8, 1951. P.S.E.B.M., 1951, v76.

Ascorbic Acid Concentration in the Adrenals of Lymphosarcoma-Bearing Mice.* (18478)

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Savard(1) has described recently a progressive reduction in ascorbic acid concentration in the adrenals of female Carworth Farm mice during the growth of a transplanted tumor (sarcoma 180). The lymphocyte is known to be an end cell of pituitary-adrenal cortical secretions, and the possibility that the products of lymphocyte destruction may exert a suppressive influence on this endocrine system has been noted in a recent publication(2). It seemed of interest, therefore, to investigate adrenal ascorbic acid levels in mice bearing a rapidly growing lymphosarcoma. The present report describes these observations as well as incidental observations concerned with a sex difference in the responsiveness of adrenal ascorbic acid to the administration of histamine and adrenocorticotrophic hormone (ACTH).

Methods. Male and female mice of the CBA strain (Strong) 8 to 10 weeks of age were used. Male mice were inoculated subcutaneously with a small fragment of lymphosarcoma(3) and were autopsied at varying intervals thereafter. Histamine acid phosphate (Abbott) was administered intraperitoneally as a solution in normal saline in doses ranging from 0.25 to 5.0 mg of histamine base per 100 g body weight. One mg of ACTH (Armour) was administered intraperitoneally as a fresh solution in distilled water. All intraperitoneal injections were given in a volume not exceeding 0.5 ml and all mice were sacrificed 1 hour following the administration of either substance. The determination of ascorbic acid in the adrenals of fasted mice was made at the end of a 48-hour period during which mice were maintained in individual metabolism cages with free access to water and without food. Im-

* This investigation was aided by a grant from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

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3. Gardner, W. U., Dougherty, T. F., and Williams, W. L., *Cancer Research*, 1944, v4, 73.

TABLE I.* Concentration and Total Content of Ascorbic Acid in Adrenals of Male CBA Mice Carrying a transplantable Lymphosarcoma.

Group	No. of mice	Tumor wt, mg	Body wt, g	Adrenal wt†	Ascorbic acid conc.‡	Total adrenal ascorbic acid, μg
Normal	25	—	22.8 ± 0.6	12.9 ± 0.4	235 ± 5	6.72 ± 0.15
Tumor 6 days‖	6	0	23.6 ± 1.6	13.3 ± 1.9	191 ± 11¶	6.42 ± 0.28
11 " ‖	8	100	24.5 ± 1.5	11.8 ± 0.9	229 ± 8	6.51 ± 0.46
14 " ‖ to 15 " ‖	8	500 to 3000	23.3 ± 1.9	12.4 ± 0.8	214 ± 15	7.14 ± 0.49
21 " ‖	6	1000 to 3000	25.9 ± 1.2	12.6 ± 1.6	230 ± 16	6.84 ± 0.40
Tumor§ (6-21 days)	28	0 to 3000	24.2 ± 0.6	12.2 ± 0.5	224 ± 6	6.57 ± 0.20

* Values shown are means and standard errors.
† mg of adrenal per 100 g body weight.
‡ mg of ascorbic acid per 100 g fresh adrenal.
§ Pooled values for all intervals after tumor implantation.
‖ Represents days after inoculation of tumor transplant.
¶ Significantly lower than normal value ($p = <0.01$) by "weighted" t-test.(7)

TABLE II.* Ascorbic Acid Concentration and Total Content in Adrenals of Normal and Lymphosarcoma-bearing CBA Male Mice Following a 48-hour Fast.

Group	No. of mice	Body wt	Adrenal† wt	Ascorbic acid conc.‡	Total adrenal ascorbic acid, μg
Normal, fed	25	22.8 ± 0.6	12.9 ± 0.4	235 ± 5	6.72 ± 0.15
Tumor (6-21 days)§ fed	28	24.2 ± 0.6	12.2 ± 0.5	224 ± 6	6.57 ± 0.20
Normal, fasted	10	20.2 ± 0.5	15.2 ± 0.6	247 ± 6	7.53 ± 0.30
Tumor (9-15 days)§ fasted	12	22.4 ± 0.9	15.7 ± 0.7	200 ± 10‖	6.93 ± 0.40

* Values shown are means and standard errors.
† mg per 100 g body weight.
‡ mg per 100 g fresh adrenal.
§ Days following inoculation of tumor transplant.
‖ Significantly lower than mean value for normal fed group ($p = <0.01$) by "weighted" t-test.(7)

mediately after sacrifice of each mouse, both adrenals were removed, trimmed of fat and connective tissue on saline-moistened filter paper, weighed to the nearest 0.1 mg on the Roller-Smith torsion balance, and extracted in a mortar with 3 ml of 6% trichloroacetic acid. The ascorbic acid content of a 2 ml aliquot of this extract was measured with the aid of a Coleman Jr. spectrophotometer by the method of Roe and Kuether(4).

Results. As is evident from the data of Table I, no progressive or consistent depletion of ascorbic acid was observed in the adrenals of male mice autopsied at varying intervals after the implantation of lymphosarcoma tissue. At only 1 interval (6 days), at a time when the tumor had not yet become grossly visible even at autopsy, was there a statistically significant, although small, drop in adrenal ascorbic acid concentration below the control value. At this interval the mean value for total micrograms of adrenal ascorbic acid is not significantly different from the control. At all other intervals, even late in the course of the tumor, when the mice were moribund, the values both of adrenal ascorbic acid concentration and total content were

4. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.

TABLE III.* Adrenal Ascorbic Acid Response to Histamine and ACTH in Male and Female CBA Mice.

Group	No. of mice	Body wt	Adrenal† wt	Ascorbic acid conc.‡	Total adrenal ascorbic acid, μg	Total adrenal ascorbic acid, μg/100 g body wt
			Males—mg histamine per 100 g body wt.			
Control	25	22.8 ± 0.6	12.9 ± 0.4	235 ± 5	6.72 ± 0.15	29.2 ± 1.0
.25 & .50	4	21.2 ± 1.7	14.2 ± 0.7	202 ± 13	6.04 ± 0.32	29.3 ± 3.4
.75	6	23.9 ± 1.5	13.5 ± 1.6	193 ± 8§	6.15 ± 0.30	26.4 ± 2.6
1.0	6	24.8 ± 1.5	12.9 ± 0.8	182 ± 12§	5.80 ± 1.00	23.4 ± 1.1§
2.0	1	21.6	11.6	138	3.45	16.0
5.0	1	22.0	13.6	175	5.25	23.9
ACTH (1 mg)	4	21.5 ± 1.1	14.4 ± 1.0	142 ± 7§	4.35 ± 0.26§	20.0 ±.1.0§
			Females—mg histamine per 100 g body wt.			
Control	9	20.0 ± 1.2	23.4 ± 1.1	234 ± 8	11.9 ± 0.2	54.8 ± 1.8
1.0	2	17.9	24.1	222	9.5	53.5
2.0	8	23.1 ± 1.3	21.2 ± 0.8	222 ± 10	10.9 ± 0.6	47.1 ± 3.3
5.0	4	24.3 ± 1.5	20.1 ± 1.1	201 ± 10	9.8 ± 0.8	40.0 ± 2.0§
ACTH (1 mg)	4	23.1 ± 0.9	21.3 ± 2.0	199 ± 5	9.7 ± 0.5	42.2 ± 3.6

* Values are means and standard errors.
† mg per 100 g body weight.
‡ mg per 100 g fresh adrenal.
§ Significantly lower than appropriate control value ($p = <0.01$) by "weighted" t-test.(7)

remarkably similar to those of the control, tumor-free group.

In connection with observations of another nature, analyses of adrenals for ascorbic acid were also made in mice following a 48-hour fast (Table II). No significant change in concentration or total content of adrenal ascorbic acid was demonstrated, in confirmation of previously reported studies in fasted rats (5). However, a trend toward an increased total quantity of ascorbic acid was observed in the adrenals of fasted normal animals. On the other hand, it is of interest that the combined stress of a rapidly-growing tumor and a 48-hour fast resulted in a significant reduction in the concentration of adrenal ascorbic acid.

In Table III are presented data concerned with the response of adrenal ascorbic acid to the administration of varying doses of histamine and of 1 mg of ACTH. In agreement with data for the rat published by Sayers and Sayers(6), it will be noted that a progressive depletion of adrenal ascorbic acid results from the intraperitoneal administration of increasing doses of histamine to male mice.

The refractivity of the adrenal ascorbic acid concentration of female CBA mice both to histamine and ACTH is also evident in the data of Table III. Histamine at a dosage-level of 0.75 mg of histamine base per 100 g of body weight was sufficient to produce a significant reduction of adrenal ascorbic acid concentration in the male mouse. In contrast, intraperitoneal doses of histamine base as high as 5.0 mg per 100 g of body weight administered to female mice failed to produce a significant drop in the concentration of adrenal ascorbic acid, although a significant fall in the total content of adrenal ascorbic acid per unit body weight was demonstrable following this dose. Similarly, 1 hour after the administration of 1 mg of ACTH to each of a group of male and female mice, the mean concentration value of adrenal ascorbic acid had fallen 40% in the males and only 19% in the females, from the appropriate control values.

Since concentration values for adrenal ascorbic acid are closely similar in male and female mice, while the weight of both adrenals in the female is roughly twice that in the male, it is evident that the female mouse possesses a total quantity of adrenal ascorbic acid approximately twice that of the male. It is of

5. Sayers, G., and Sayers, M. A., *Recent Progr. Hormone Research,* 1948, v2, 81.

6. Sayers, G., and Sayers, M. A., *Endocrinology,* 1947, v40, 265.

7. Snedecor, G. W., Statistical Methods, Iowa State College Press, Ames, 1946, p. 83 ff.

interest to note that when a comparison is made of the total quantity of ascorbic acid disappearing from the adrenals of male and female mice following the administration of ACTH, the respective values are closely similar—2.4 and 2.2 micrograms in the case of males and females, respectively. A similar relationship with respect to adrenal ascorbic acid values is suggested following the administration of larger doses of histamine, although the data for corresponding doses in males and females are insufficient to establish this point.

Discussion. The data described in this study demonstrate that the adrenal ascorbic acid response of the host to the growth of a tumor(1) is not necessarily a general phenomenon independent of such variables as species, sex, strain and tumor type. When it occurs, the depletion of ascorbic acid in the adrenal, generally recognized as a consequence of severe stress(5), may be associated not with the growth of a given tumor *per se* but with incidental nonspecific tissue changes such as inflammation or necrosis. The failure to demonstrate lability of adrenal ascorbic acid in an animal with a rapidly growing lymphosarcoma is of some interest in connection with the possibility mentioned previously that a reciprocal relationship may exist between lymphoid tissue and the pituitary-adrenal cortex system. This hypothesis, however, cannot be even tentatively supported solely from these data, unless further observations show lymphoid tumors to be unique in this respect.

It may be suggested that the growth of the tumor studied represents a stress of subthreshold effectiveness, since when combined with a period of fasting, significant reduction of the adrenal ascorbic acid concentration results, although neither stimulus is effective alone.

Finally, the data concerning differences in the response of male and female mice to graded chemical stresses suggest that whatever the role of ascorbic acid in the production or release of adrenal cortical steroids, its utilization expressed as the disappearance of an actual quantity of ascorbic acid from the gland has a more constant quantitative relationship to the stimulus than when expressed as a given decrement in its concentration.

Summary. The progressive depletion of ascorbic acid previously reported to occur in the adrenals of female mice carrying a transplantable sarcoma has not been confirmed in the case of male mice of another strain bearing a transplantable lymphosarcoma. A pronounced sex difference exists in the adrenal ascorbic acid response of CBA mice to histamine and ACTH, although relatively constant total quantities of ascorbic acid may disappear from the glands of each sex following similar stimuli.

Received January 9, 1951. P.S.E.B.M., 1951, v76.

Nutritional Factors in Hemodynamics: Dissociation of Pressor Response and Hemorrhage Resistance in Avitaminosis C.* (18479)

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Direct microscope observations of the mesenteric capillary bed in vit. C deficient guinea pigs have revealed that in addition to slowed flow and "increased fragility" of the collecting venules there was also present a reduced reactivity of the terminal arterioles and precapillary sphincters to topically applied epinephrine(1). This latter phenomenon suggested that ascorbic acid was perhaps one

* This study was supported in large part by the Nutrition Foundation, New York. We are also grateful to the Upjohn Company of Kalamazoo, Mich., for supplies of certain vitamins.

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of the factors essential for the functioning of certain peripheral vasomotor mechanisms. Kasahara and co-workers(2,3) have described reduced pressor responses to intravenously injected epinephrine in guinea pigs receiving scorbutogenic diets. These reports have been substantiated by Freire(4). In addition, the perfused hind-limbs of scorbutic guinea pigs are reported to have a notably diminished vascular response to the vasoconstrictor effects of epinephrine(5). These findings could perhaps be explained at least in part by an epinephrine-protecting action of ascorbic acid contained either in the blood or the perfusion fluid in the control observations. This antioxidant property of the intravascular vit. C presumably would have little if any direct influence, however, on the peripheral vascular responses to epinephrine topically applied to the outside of the vessels observed. The direct method of testing epinephrine responsiveness therefore offered possibly more convincing evidence suggestive of vasomotor impairment in avitaminosis C. To supplement this evidence, we have examined the ability of vit. C deficient guinea pigs to vasocompensate in response to vascular stress. In addition, comparisons were made between the mean blood pressures and the pressor responses to intravascular injections of epinephrine prior to the stress period, in the deficient and in the supplemented animals. It was found that although the initial mean blood pressure and its rise following epinephrine (at the dosage used) were not significantly affected by vit. C deficiency, the capacity to withstand hemorrhage in such animals was greatly diminished.

Methods. Nineteen guinea pigs of 500-600 g body weight were given a synthetic ration free of ascorbic acid.‡ Ten of these animals received daily intraperitoneal injections of vit. C, 10 mg per 100 g body weight, neutralized with 0.5% sodium bicarbonate. Nine of these supplemented animals were pair-fed with the remaining nine guinea pigs who received no ascorbic acid. This program was continued for 24-26 days, then the following procedures were carried out on each animal. General anesthesia was induced with sodium pentobarbital (3 mg/100 g body weight) and the carotid artery was cannulated with a blunt No. 22 needle. Coagulation of blood was prevented by a previous intramuscular injection of Heparin, 100 units. The initial blood pressure and its rise following intravenous (or intra-arterial) epinephrine, .001 mg/100 g body weight, was recorded, using a Hurthle membrane manometer. Following the return of normotension, blood was slowly withdrawn from the carotid cannula over a 4-5 minute period, until the blood pressure had fallen to approximately 60% of its initial level. At intervals of 30 minutes, blood samples of 2 cc/500 g body weight were taken until the animal expired. The plasma from certain samples was refrigerated at 7°-8°C for 24 hours and was then tested for vasotropic properties by the Chambers-Zweifach rat meso-appendix test(6,7).

Results. It was noted early in the study that guinea pigs not supplemented with ascorbic acid were unusually susceptible to the hypotensive action of the anesthetic used. In the controls, satisfactory complete surgical anesthesia was obtained using 3 mg of sodium pentobarbital/100 g body wt, injected intramuscularly. The deficient animals were adequately anesthetized without relative hypo-

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‡ This diet was composed of Vit. Test Casein—18%; cornstarch, 49%; lard, 5%; sucrose, 15%; salts (U.S.P. 12 salt mixture No. 2), 4%; CLO, 1%; WGO, 2%; corn oil, 1%; and vitamin supplements, 1%. These latter were (per 100 g diet): Thiamine HCL, .8 mg; Riboflavine, .8 mg; Pyridoxine HCL, .8 mg; cal. pantothenate, 1.5 mg; nicotinic acid, 1.5 mg; nicotinamide, 1.5 mg; choline chloride, 400 mg; and Inositol, 100 mg.

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7. Zweifach, B. W., In Methods in Medical Research, 1948, v1, 131-146; Yearbook Publishers, Inc., Chicago, Ill.

TABLE I. Blood Pressure and Pressor Responses, Blood Loss, and Plasma Vasotrophic Activity in Rat Test of Representative Animals from Supplemented and from Deficient Groups. Findings in remaining animals are in close accord with the data given.

No.	Initial B.P. (mm Hg)	B.P. level after epinephrine	Initial hemorrhage (cc/500 g body wt) cc	Total amt of blood loss (cc/500 g body wt) cc	Survival time, min.	Rat assay of plasma 1, 2		
						Sample 1 (30 min.)	Sample 2 (60 min.)	Sample 3 (90 min.)
				Supplemented				
1	100	120	7	14	34	grde 1 VD	—	—
2	70	120	8	20	80	neutral	grde 1 VE	grde 3 VE
3	100	140	5	12	100	neutral	grde 1 VE	grde 3 VE
4	65	130	10	17	100	neutral	grde 2 VE	grde 3 VE
5	80	220	8	20	130	neutral	grde 2 VE	grde 3 VE
6	80	134			110			
	Avg 83	146	7.6	16.6	92.0			
				Deficient				
1	84	135	5	7	50	lost	grde 1 VD	—
2	52	145	3.5	3.5	10	grde 1 VD	—	—
3	78	122	4	8	30	grde 2 VE	—	—
4	70	120	5	6	8	neutral	—	—
5	95	139	4	8	48	lost	neutral	—
6	82	190	5	11	90	neutral	grde 1 VD	grde 1 VD
	Avg 77	143	4.3	7.1	39.0			
Table of x P = 0.52			<0.01	<0.01				

1. A dash indicates that no sample was obtained because of previous death of the animal.
2. VE = vaso-excitor; VD = vaso-depressor.
3. If the animal's death became imminent the sample was drawn immediately.

tension with approximately 1-2 mg of the agent/100 g body weight. Larger doses were frequently followed by a rapid and severe fall in blood pressure and a subsequent respiratory failure. The pigmented animals receiving the unsupplemented diet seemed possibly more susceptible than the albinos to this vaso-depressor property of the anesthetic agent.

Following anesthesia, the mean blood pressure levels before bleeding were slightly below those found in the control group (Table I). The p value of the difference, however, was greater than 0.5. Under these conditions, therefore, no significant difference existed between the mean blood pressures of the control and the deficient animals. Epinephrine injections were followed in all cases by rises in blood pressure that were comparable both in rate and in degree to those of the controls.

The deficient animals were not as capable as were the controls in withstanding blood loss. The removal of 5-10 cc/500 g body wt reduced the blood pressure of animals on a supplemented diet to approximately 60% of its normal level. Comparable amounts of blood withdrawn from non-supplemented individuals invariably lowered the pressure to about 25-35% of normal, and in some instances, was followed by a rapid death of the animal. For this reason, it was found necessary to remove only 3-5 cc of blood from them in order to induce the desired hypotension. The p value of the difference is less than .01 (Table I). In addition to this susceptibility to the initial hemorrhage, further blood loss by sampling was also poorly withstood by the deficient group (Fig. 1). They occasionally succumbed shortly after withdrawal of the first (30 min.) blood specimen, in contrast to the facility with which control animals readily endured repeated removal of blood. The average survival time of the deficient animals was 39 min., in contrast to 92 min. for the supplemented controls. This susceptibility to subsequent hemorrhage was also accompanied by a relative reduction in the total amount of blood obtained from the deficient animals (Table I).

Rat meso-appendix assays were made of the plasma samples obtained from 8 supplemented animals following the onset of hypotension.

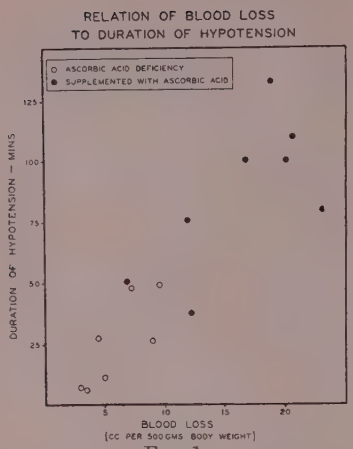

FIG. 1.

The total volume of blood loss is plotted against the duration of survival at hypotension for deficient and supplemented animals. It is evident that avitaminosis C is accompanied by a reduction of both features.

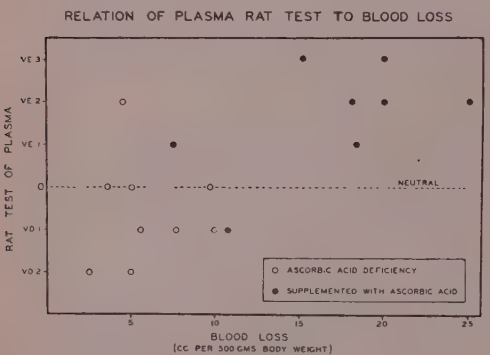

FIG. 2.

The vasotropic properties of the final plasma sample obtained from each animal is compared graphically to the total volume of blood loss endured. The relative impairment in ability to withstand hemorrhage of the ascorbic-acid deficient animals is associated with plasma vasoactivity of predominantly vaso-depressor quality. (VE = vaso-excitor; VD = vasodepressor; graded in increasing magnitudes of 1, 2, and 3, from the "neutral" baseline at zero across the center of the figure).

The first sample from animals No. 2-9 was neutral, but that of No. 1 was slightly vaso-depressor. This animal was one which had failed to adapt well to the diet, and it expired shortly after the sample was withdrawn. The second sample (at 60 min. of hypotension) was slightly to moderately vaso-excitor in character in No. 2, 3, and 5, and still neutral in No. 3, 7 and 8. The third plasma sample (90 min. of hypotension) was strongly vaso-excitor in all surviving animals.

In sharp contrast to plasma of the control animals, the plasma from deficient animals, with one exception, showed a complete absence of vaso-excitor activity in the rat test (Table I) (Fig. 2). Instead, despite the brief duration of their hypotension and only moderate blood loss, the plasma of many of the animals which survived a period sufficiently long to permit a 60 min. sample was vaso-depressor in character. The one deficient animal with vaso-excitor properties prevalent in the plasma samples was also very resistant to anesthesia (ultimately it received 3x the usual dose) and had no recognized symptoms of vit. C deficiency.

Discussion. It is of interest that with poor resistance to hemorrhage, plasma samples from the deficient animals did not exhibit the vaso-excitor property in the rat test (contain VEM) that was uniformly observed in the plasma of control animals under apparently similar circumstances. During and subsequent to such procedures as hemorrhage, trauma, etc., the elaboration of VEM is considered possibly to constitute a humoral vaso-compensatory process, probably supplementing neurogenic vasoconstrictor tone(8). It is known that the presence of vaso-excitor material is dependent at least in part on adrenal cortical secretion(9). Vit. C deficiency may prevent VEM formation or release by interfering with the availability of adrenal factors or perhaps other essential substances. Disturbances in VEM production are not related to one specific dietary deficiency, however. Payne and her co-workers have shown conclusively that rats on a low protein diet are relatively incapable of forming this principle(10).

Greatly reduced or absent pressor responses to intravenous injections of epinephrine in vit. C deficient guinea pigs have been de-

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10. Payne, M. A., and Shorr, E., *Fed. Proc.*, 1949, v8, 125.

scribed by several workers(3,4). Our data revealed no essential differences between the deficient and the control groups, with regard either to rate or degree of such a pressor reaction. The dose of epinephrine used in this study, however, was somewhat greater than that used by the previous workers. This undiminished pressor response to epinephrine of the deficient animals contrasted greatly with their impaired capacity to compensate for blood loss. This finding militates strongly against the use of such acute pressor tests as attempts to predict vaso-compensatory capacity, at least in response to hemorrhage. It is not known whether such a similar dissociation exists between the ability to raise blood pressure in response to epinephrine injections and the power to resist or survive other prolonged hypotensive procedures such as trauma, burns, etc.

Previous direct microscopical observations of the capillary bed in avitaminosis C revealed undiminished constrictor responses to topical epinephrine in the larger more proximal arterioles (greater than 90-100 μ in diameter). This reactivity of the metarterioles, however, (terminal arterioles supplying the capillary bed directly) was greatly diminished(1). It is conceivable that these observations are related to the apparent dissociation between acute pressor ability and the capacity to resist hemorrhage in vitamin C deficiency. The vasoconstrictor component in pressor response of *acute* and transient nature may depend in large part upon the maintained responsiveness of these larger arterioles. If this be true, then what is the role, if any, in the pressor mechanism, of the smaller terminal arterioles? Their *hypo*-reactivity to epinephrine, along with other phenomena, accompanies reduced resistance to hemorrhage. On the other hand, in such conditions as early shock, and experimentally induced hypertension, these arterioles in animals on fully supplemented diets become greatly and persistently narrowed, and gain and maintain an *augmented* reactivity to epinephrine. This state is believed to be mediated in large part by humoral factors, and perhaps to serve during vascular strain as an important vaso-compensatory mechanism(8). Thus by supplementing the peripheral resistance produced by the larger vessels, the capacity for vasoconstriction in these smaller terminal arterioles may be of relatively greater importance in effecting more *sustained* attempts at blood pressure elevation.

Conclusions and summary. 1. As compared to pair fed controls, guinea pigs on a vit. C free diet showed no obvious differences in blood pressure or in ability to respond in pressor manner to intravenous or intra-arterial injections of epinephrine at the doses employed. 2. The animals were bled by a standard procedure of hemorrhage. Avitaminosis C was accompanied by a significant reduction in the survival time, in resistance to anesthesia, and in the ability to withstand comparable amounts of blood loss. In addition, all deficient animals, with one exception, were unable to elaborate renal VEM as determined by rat assays of the plasma. 3. The significance of these observations is discussed. The suggestion is offered, in relation to earlier findings, that the larger arterioles with undiminished constrictor responses to epinephrine in avitaminosis C may function primarily in maintaining normotension and in producing the vasoconstrictor component of *acute* pressor reactions. The smaller, terminal arterioles, with greatly diminished epinephrine reactivity during vitamin C deficiency, may act chiefly in the pressor mechanism to supplement the larger vessels in acute stress states and during *prolonged* attempts at blood pressure elevation.

Received January 12, 1951. P.S.E.B.M., 1951, v76.

Preparation of a Stable Non-Infective Soluble Influenza A Antigen. (18480)

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The soluble antigen of influenza A (PR8) prepared from the specifically infected chorioallantoic membranes(1) is an excellent antigen in the complement-fixation test for the laboratory diagnosis of influenza A. This soluble antigen can be rendered non-infective by the addition of formalin(2) or by ultraviolet irradiation(2,3). Ultraviolet irradiation requires special apparatus(4,5) while the use of formalin has been found to render the antigen anti-complementary on storage and unsuitable for lyophilization since the reconstituted material has little or no antigenicity.

In an attempt to develop a simple method of preparing a non-infective soluble influenza antigen that would remain stable on long storage, two procedures were developed. One is based on the use of hydrogen peroxide, the other on the use of formalin followed by its neutralization prior to storage or lyophilization.

Materials and methods. The soluble antigen was prepared from the chorioallantoic membranes of 13-day-old chick embryos which had been infected with the PR8 strain of influenza A virus by the allantoic route 2 days previously. To the pooled membranes was added 2.5 ml of saline per membrane and the mixture ground for 2 minutes in a Waring blendor. After a preliminary centrifugation for 10 minutes at 2000 r.p.m., the supernatant suspension was centrifuged for 1 hour at 15,000 r.p.m. The supernatant liquid was removed and used as the soluble antigen. It was tested for hemagglutinins by the procedure of Salk(6) and found to contain less than 20 units per ml. The ID_{50} was $10^{-7.2}$ when titrated in the allantoic cavity. Infectivity tests of control and treated samples were conducted by direct inoculation of undiluted and 10^{-2} diluted solutions into the amniotic sac of 11-12-day-old chick embryos and a subsequent incubation for 72 hours. Samples which after 2 successive amniotic passages yielded amniotic and allantoic fluids free of specific hemagglutinins were considered to be non-infective. Preliminary experiments had shown that this soluble antigen could be clarified by treating with Norit (Norit "A" from Fisher Scientific Co.) or certain concentrations of hydrogen peroxide (Eimer & Amend, reagent 30%) with little or no loss of the antigenicity. Although the Norit-treated fractions were still infective, some of the peroxide-treated ones were not. The following experiment was conducted to determine the optimal concentration of peroxide and experimental conditions to yield a soluble antigen which was non-infective, could be lyophilized, stored and then redissolved with a minimal loss of antigenicity.

Three temperatures, 0°, 22° and 37°C were selected for the initial peroxide-inactivation reaction. Aliquots of a common pool of the soluble antigen were placed in cotton stoppered Erlenmeyer flasks in water baths at the above temperatures. After 10 minutes, hydrogen peroxide was added to the antigens in the proportion of 1:10 to give the final peroxide concentrations shown in Table I. Normal membrane antigen was treated similarly as a control. After a given reaction period at the designated temperature, the flasks were placed in a cold room at 4°C overnight. To remove the excess hydrogen peroxide, the solutions were then shaken with 3% by weight of Norit at room temperature

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TABLE I. Treatment of PR8 Soluble Antigen with Hydrogen Peroxide.

Antigen content of H_2O_2, %	Reaction temp., °C	Reaction time, hr	CF titer	Infectivity	After lyophilization: CF titer	After lyophilization: Infectivity
0 (control)	0	0	64	+	64	+
3	0	2	32	+	32	+
3	22	½	8	—	<2	
3	37	½	2	—	<2	
2	0	2	64	+	64	+
2	22	2	64	+	64	
2	37	½	8	—	4	
1	0	2	64	+	64	+
1	22	1	64	+	64	+
1	37	1	32	+	32	+
1 then increased to 2	37	1	32	—	32	
2	22	1				

for one hour, alternately applying and removing a vacuum. The solutions were centrifuged for 5 minutes at 5000 r.p.m. and the clear supernatant liquids were removed. The antigenicity of these liquids was determined in the complement-fixation test(7), using 2 units of complement and 4 units of amboceptor. Aliquots were lyophilized, reconstituted to their original volume with distilled water and also tested. All samples were then tested for infectivity as described.

Although formaldehyde is a well-known virucidal agent, it was found that when the concentration of formaldehyde present in the soluble antigen was sufficient to destroy infectivity, lyophilization of the antigen for storage produced an almost insoluble material, suggestive of protein denaturation, which was no longer antigenic. It therefore seemed obvious that if, after the period of virus inactivation with formaldehyde, the excess formaldehyde could be removed or neutralized chemically, the lyophilization process might be more successful. Ammonia and ammonium salts are highly effective in converting formaldehyde to hexamethylenetetramine(8), especially at a neutral or slightly alkaline pH. Therefore, after the treatment of the antigen with formaldehyde, the amount of 5% ammonium hydroxide theoretically required from the equation:

$$6\ CH_2O + 4\ NH_3 \longrightarrow (CH_2)_6N_4 + 4\ H_2O$$

to convert all the formaldehyde to hexamine was added. Since some of the formaldehyde is consumed in reaction with the virus and protein, a slight excess of ammonia is present which is desirable for the conversion of formaldehyde to hexamine. Formaldehyde solution was added to the antigen in the proportion of 1:10 to give the final concentrations shown in Table II. The treated antigens were held at the designated temperatures for 2 hours and then placed in the cold room at 4°C overnight (16 hours). The excess formaldehyde in aliquots of each sample was neutralized by the addition of the theoretical amount of 5% ammonium hydroxide, the solutions maintained at 25°C for 1 hour and then the pH adjusted to 7.2-7.4 with 0.1 N sulphuric acid or isotonic dibasic sodium phosphate.

Aliquots were lyophilized and then redissolved to the original volume with distilled water. After testing for antigenicity and infectivity, samples were stored at 4°C in sealed

TABLE II. Treatment of PR8 Soluble Antigen with Formaldehyde.

% HCHO	Temp., °C	CF titer	Infectivity	After lyophilization: CF titer	After lyophilization: Infectivity
0 (control)	25	16	+	16	+
.5	25	32	—	<2	
.5 + NH_3	25	8	—	8	
.25	25	16	—	<2	
.25 + NH_3	25	8	—	4	
.1	25	16	—	<2	—
.1 + NH_3	25	16	—	16	—
.1	37	16	—		
.1 + NH_3	37	16	—		
.03	25	16	+	<2	
.03 + NH_3	25	16	—	16	

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ampoules or lyophilized. The results by this procedure are shown in Table II.

As a final check on the virus inactivation by this procedure, a fresh lot of the soluble antigen was prepared in the usual manner. One aliquot of it was deliberately contaminated with PR8 virus by adding to it the pellet containing the virus which was obtained by centrifuging an equivalent volume of infected allantoic fluid. The soluble antigen had a hemagglutination titer of less than 20 units per ml and the ID_{50} was $10^{-6.5}$ whereas the antigen plus virus had a hemagglutination titer of 2560 units per ml and the ID_{50} was $10^{-8.7}$. The samples were then treated with 0.1% and 0.15% formaldehyde, both with and without subsequent neutralization. Infectivity tests showed all samples to be non-infective and the neutralized samples had lost no antigenicity before or after lyophilization.

At the time of writing, samples of antigens treated with hydrogen peroxide or formaldehyde plus neutralization have been stored for over 2 months in sealed ampoules and in the dried condition at 4°C and have lost none of their antigenicity nor have they become anti-complementary.

Discussion. It can be seen from Table I that (1) when the soluble influenza A (PR8) antigen contained 3% hydrogen peroxide, the infectivity was destroyed only under conditions which caused a large loss of the antigenicity (2) treatment with 2% peroxide at 22°C for 2 hours, followed by lyophilization, produced a non-infective antigen with little or no loss of antigenicity (3) treatment with 1% peroxide at 37°C for 1 hour followed by an increase to 2% peroxide at 22°C for 1 hour yielded an antigen which was non-infective after lyophilization and which had lost little or none of its antigenicity. In the 3 experiments done, this antigen was also non-infective prior to lyophilization.

From Table II it is evident that when the concentration of formaldehyde in the antigen is sufficient to destroy the infectivity, lyophilization destroys the antigenicity. However, when the excess formaldehyde is neutralized with ammonia prior to lyophilization, the antigenicity remains intact. The fact that the reaction temperature can be increased to 37°C for 2 hours and the ammonia concentration to pH 10 without loss of the antigenicity indicates that the antigen is quite stable to this method of treatment. Normal membrane antigen treated by this procedure gave no significant nonspecific antigen titer in the complement-fixation test.

Since the antigenicity is more stable to variations in temperature and reagent concentration with the formaldehyde treatment, it is considered to be the better method.

Summary. Two methods for the preparation of a stable non-infective soluble influenza A antigen have been developed. One is based on treatment with 2% hydrogen peroxide, the other on treatment with 0.1% formaldehyde followed by its neutralization prior to storage or lyophilization.

Received January 13, 1951. P.S.E.B.M., 1951, v76.

In Vitro Cultivation of the Parasitic Phase of *Coccidioides immitis*. (18481)

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To date only partially successful results have been obtained in attempts to culture the parasitic phase of *Coccidioides immitis* which exists as a spherical endospore-producing organ (commonly known as 'spherule') *in vivo* and as a fluffy white mold *in vitro*.

* The author wishes to thank Prof. W. H. Weston for aid and counsel during the course of this work; Miss Betty Insley and Dr. G. Morel for tubes of coconut milk agar medium; Dr. E. E. Baker for suggestions concerning the research, and Dr. N. F. Conant for supplying 5 strains of *C. immitis*.

Continued multiplication of the spherules for as long as 3 weeks was noted by MacNeal and Taylor(1) when pus containing the organisms was added to ascitic fluid or to gelatinized horse serum containing sterile kidney slices. The *in vitro* transformation of culture-grown chlamydospores into spherules was noted first by Lack(2) who used Hall tubes containing glucose broth and partially coagulated egg albumin in which chlamydospores were suspended. Baker and Mrak(3) reported 2 out of 16 strains of *C. immitis* to produce *in vitro* structures closely resembling spherules. These structures, called "culture spherules," were produced under aerobic conditions at room temperature and 37°C and were found to develop on a variety of media.

The present paper reports the *in vitro* formation of spherules, or sporangia as they are more properly termed, by all of 7 representative strains of *C. immitis* tested: strain VA 10 was isolated by Mrs. Lily Anisman, and strain VA 7 was isolated by the author at the Veterans Adm. Hosp., Sawtelle, Los Angeles, Calif. Strains 660 (Argentina), 2200 (Ala., pt. in Ariz. 4 years previously), 2150 (Texas), 2153 (Ariz.), and 2191 (Ga., pt. in Calif. 10 years previously) were furnished by Dr. Norman F. Conant, Duke Univ. Med. School, Durham, N. Carolina. The fungi were cultured at room temperature at 37°C on the following medium: coconut milk 250 ml; Knop Sol., ½ dil. 500 ml; dist. H_2O 250 ml; trace elements Berthelot sol. 10 drops; dextrose 3%; washed agar 1%; cysteine 10^{-6}; thiamine 10^{-5}; and naphthalene acetic acid 10^{-7}. The chemically defined portion was autoclaved and cooled before adding the coconut milk which had previously been sterilized by filtration. Tubes or flasks of this medium were inoculated on the surface with the mycelium and chlamydospores of *C. immitis* and were incubated at room temperature and at 37°C.

Although growth was evident in 2-3 days, the fungus developed slowly, most of the mycelium being below the surface. The formation of aerial mycelium or of compact leathery mats was prevented by folding such colonies into the medium. Eventually, the fungus produced a soft, grey to tan subsurface growth in which the sporangia developed. Sporangia could be examined microscopically by removing a portion of the growth and mounting it in a drop of lactophenol cotton blue. In such preparations the immature and mature sporangia were found to stain an intense blue in contrast with non-fertile sporangial initials which stained more lightly. In the medium described sporangia were found as early as the 11th day but they usually did not appear until much later—4 to 10 weeks in some cultures. Several tubes inoculated at the same time from the same parent strain gave varying results as regards the age of the culture at which sporangia were produced as well as in the number of sporangia formed. Incubation at 37°C did not appear to hasten sporangial formation. All stages in the development of sporangia and endospores were to be found in mature cultures. Sporangia was formed terminally and intercalarily (Fig. 1, a and b). The majority tend toward a spherical shape but occasionally weirdly shaped sporangia occurred (Fig. 8). Cleavage planes (Fig. 2 and 3) were more clearly evident than is usual in those sporangia found in host tissues and in exudates. The more normal or spherical sporangia ranged in size, when mature, from 5-30 μ in diameter and contained varying numbers of endospores which were usually, but not necessarily, of similar size (Fig. 1c, 4, 5 and 6). Newly released endospores were thin-walled and sometimes vacuolated. Preliminary study indicated that, although these spores may enlarge directly to form sporangia as in host tissues, they frequently germinated to yield hyphae which, in turn, quickly formed more sporangia (Fig. 7, a and b). The thickness of the sporangial wall was found to vary: in those sporangia formed in very young cultures the walls were quite thin while in those sporangia formed in mature cultures the walls were much thicker.

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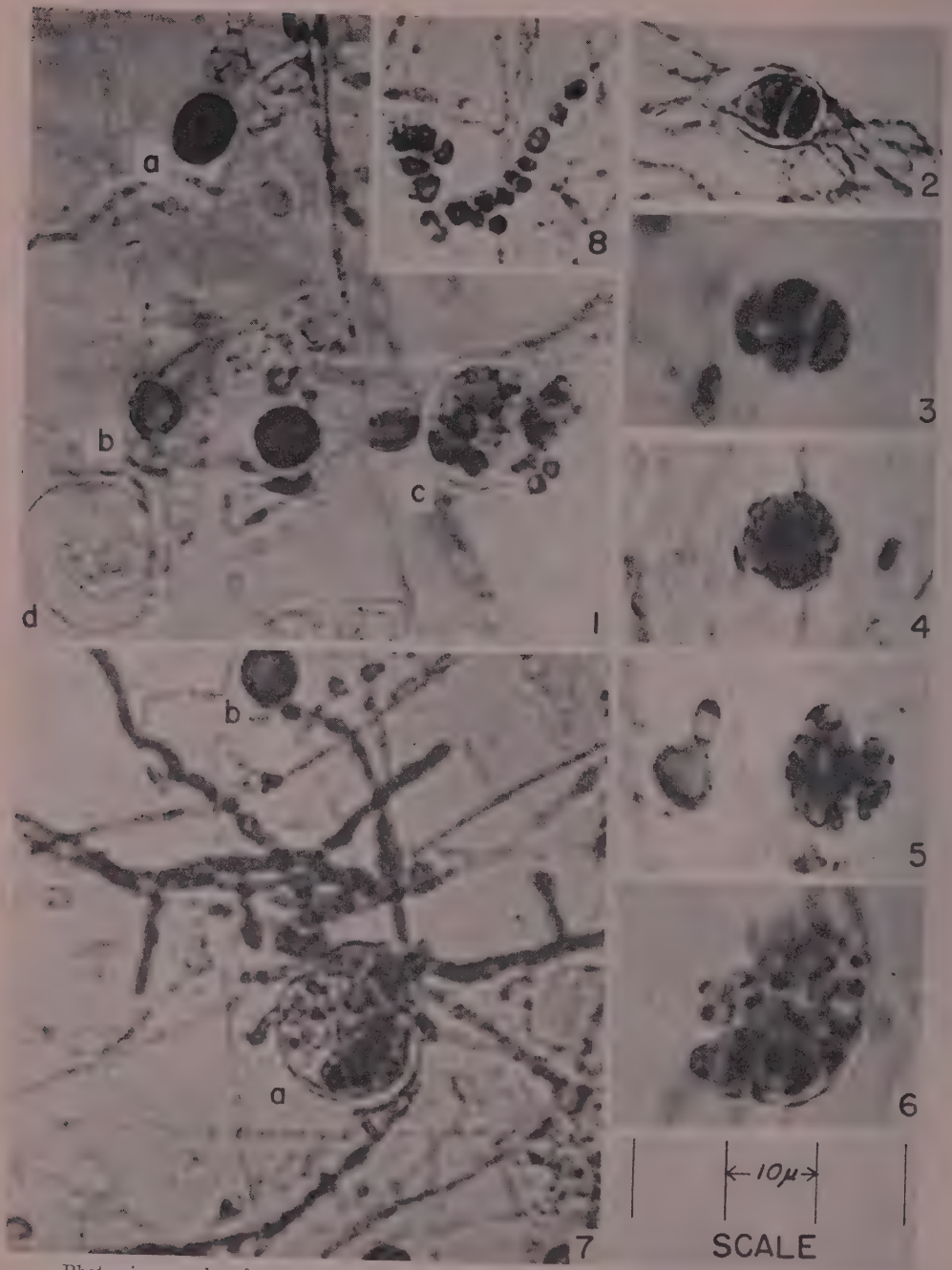

Photomicrographs of material from cultures mounted in lactophenol cotton blue (magnification approx 1300).

FIG. 1. a. Terminal sporangium. b. Intercalary sporangium. c. Mature sporangium releasing endospores. d. Empty sporangium.

FIG. 2 and 3. Early cleavage.

FIG. 4. Mature sporangium containing many small spores.

FIG. 5. Mature sporangium containing many large spores.
FIG. 6. Mature sporangium releasing endospores.
FIG. 7. a. Endospores germinating through sporangial wall. b. Immature sporangium produced by young hypha.
FIG. 8. Hyphal element serving as sporangium.

Further study on certain cytological aspects and on the exact nutritional requirements of the sporangial stage is in progress. Already it has been found that at least one strain will produce sporangia on the basic, chemically defined portion of the medium and on certain fractions thereof, though less satisfactorily than when coconut milk is added.

Summary. The *in vitro* cultivation of the parasitic phase of *C. immitis* is reported and the composition of the medium detailed. Sporangia were formed by all strains studied though in varying numbers and after varying periods of incubation. Mature culture-grown sporangia were indistinguishable from mature tissue sporangia.

Received January 15, 1951. P.S.E.B.M., 1951, v76.

Locus of the Central Emetic Action of Cardiac Glycosides.* (18482)

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It has long been generally accepted that the so-called "central" emetic drugs act by direct excitation of the vomiting center. The concept was especially advanced by Hatcher and Weiss(1) who, in acute experiments, abolished the responsiveness of cats to a variety of emetic agents by making lesions in the dorsal vagal nuclei of the medulla. Also convincing was the demonstration by these workers(1,2) that the direct application of certain "central" emetics to the region of the ala cinerea induced vomiting in dogs and cats.

The premise that drugs act directly on the vomiting center is highly contingent on the certainty of the location of this center. That the dorsal vagal nuclei do not constitute the vomiting center was first indicated by the work of Koppanyi(3). He showed that dogs with chronic lesions in the ala cinerea continued to vomit in response to orally administered irritant emetics in spite of a concomitant refractoriness to the "central" emetic, apomorphine. On the basis of prevailing thought, Koppanyi interpreted his findings as due to incomplete exclusion of the vomiting center. The location of the vomiting center and its physiological role have been adequately delineated only recently by Borison and Wang(4) and Wang and Borison(5). They have demonstrated that the vomiting center, localized in the lateral reticular formation of the medulla, is quite distinct both anatomically and physiologically from the superficial region of the ala cinerea which was shown by Hatcher and Weiss to be sensitive to direct application of certain emetics and which was erroneously designated by them

* This project was supported in part by a grant from the National Institutes of Health, U. S. P. H. S., and in part by funds contributed by Sandoz Chemical Works, Inc.

1. Hatcher, R. A., and Weiss, S., *J. Pharm. and Exp. Therap.*, 1923, v22, 139.

2. Hatcher, R. A., and Weiss, S., *Arch. Int. Med.*, 1922, v29, 690.

3. Koppanyi, T., *J. Lab. and Clin. Med.*, 1930, v16, 225.

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5. Wang, S. C., and Borison, H. L., *Arch. Neurol. and Psychiat.*, 1950, v63, 928.

as the emetic center. The question that remains is how this area in the dorsal surface of the medulla is associated with the nervous regulation of vomiting. The present report is concerned firstly with the characterization of a chemoreceptor trigger zone for emesis which is situated in the dorsal region of the ala cinerea and which serves as an afferent station for the vomiting center. Secondly, it is our purpose to show that the primary site of emetic action of intravenously administered digitalis glycosides is a specialized area in the central nervous system, namely, the chemoreceptor trigger zone for emesis.

Methods. In dogs anesthetized with pentobarbital and maintained with artificial respiration, a small bilateral lesion was placed by means of electrocautery in the caudal margin of the floor of the IVth ventricle. The emetic responsiveness of these animals was tested with apomorphine, copper sulfate and the cardiac glycosides lanatoside C, scillaren A and ouabain.† Postoperative responses were compared with the results of preoperative tests on the same dogs or with responses elicited in normal dogs given equivalent doses of the drugs. The tests were performed repeatedly during an observation period which extended for as long as 6 months postoperatively. The brains were perfused *in situ* with formalin when the animals were sacrificed, and the lesions were examined histologically.

Results. Apomorphine. Apomorphine hydrochloride was used to determine the success of the operation. A normal dog usually vomits after 0.01 mg per kg of apomorphine given intravenously and almost invariably responds to 0.02 mg per kg. Emesis occurs in about 2 minutes. In contrast, a dog in which a successful operation has been performed fails to vomit even to 1.0 mg per kg apomorphine. In approximately 1000 tests performed on more than 100 normal unanesthetized dogs, there was not a single instance when more than 0.03 mg per kg apomorphine was required to elicit vomiting. The drug was rapidly injected intravenously in 1.0 ml of a freshly prepared aqueous solution. A total of 11 dogs was subjected to the operation for destruction of the chemoreceptor trigger zone. Nine of these dogs proved to be completely and permanently refractory to apomorphine up to the limits of the doses tested, that is, 0.1 to 1.0 mg per kg.

Copper sulfate. For comparison with the "centrally" acting emetic drugs, copper sulfate was used as a gastrointestinal irritant for the purpose of eliciting vomiting through known reflex pathways(6). The oral administration of approximately 4.0 mg per kg (calculated as the anhydrous salt) copper sulfate was found to be effective for inducing vomiting in most normal dogs. In no case, in more than 100 dogs tested, was it necessary to use over 8.0 mg per kg. The copper sulfate was dissolved in 50 ml of distilled water and administered by stomach tube after one day of food deprivation. The average latent period for vomiting was 19 minutes. The 9 dogs made refractory to apomorphine by operations on the medulla did not show any significant change in responsiveness to copper sulfate. In fact, these dogs had no detectable deficit in the functional character of the vomiting in response to copper sulfate. Except for refractoriness to intravenous apomorphine, the chronic operated dogs showed no overt signs of central nervous system damage and could not be distinguished from normal dogs. Thus it has been established that the central mechanism for vomiting in response to irritation of the enteric tract by copper sulfate is intact in chronic operated dogs which are unable to respond to the very potent emetic drug, apomorphine.

Cardiac glycosides. Three cardiac glycosides, namely, lanatoside C, scillaren A and ouabain, were tested for emetic action in the dogs with lesions in the chemoreceptor trigger zone. Preliminary tests were made on normal dogs with each of the drugs to determine the emetic dose effective in all animals.

† Lanatoside C and scillaren A were kindly supplied by Mr. Harry Althouse and Mr. S. M. Fossel of Sandoz Chemical Works, Inc., and ouabain by Dr. K. K. Chen of Eli Lilly and Co.

6. Wang, S. C., and Borison, H. L., *Am. J. Physiol.*, 1951, v164, 520.

TABLE I. Emetic Activity of Certain Cardiac Glycosides Injected Intravenously.

Drug	Dose, mg/kg	Normal dogs		Operated dogs*	
		Tested	Vomited	Tested	Vomited
Lanatoside C	.05	9	3	—	—
	.07	5	2	—	—
	.08	13	13	3	0
	.10	12	12	6	0
Scillaren A	.05	6	0	—	—
	.07	8	3	—	—
	.08	5	5	—	—
	.10	5†	5	5	1‡
Ouabain	.03	3	1	—	—
	.04	3	1	—	—
	.06	3	3	2	0

* Refractory to apomorphine inj. intravenously.
† One dog died.
‡ Vomited 6 hr after inj.

The glycosides were administered by the intravenous route in a small volume of 5 to 50 per cent alcohol, each ml containing from 0.15 to 1.0 mg of the drug. When vomiting was elicited in the normal animal, the first expulsion of vomitus generally occurred within 15 minutes following injection of the drug. The data are summarized in Table I. Only one of the 9 successfully operated dogs vomited in response to doses of the cardiac glycosides shown to be effective in all normal dogs tested. This single instance of vomiting in an operated animal was in response to scillaren A and the latent period was 6 hours. Effects of the drugs at dose levels higher than those indicated in Table I were not studied in this series of experiments.

Anatomical localization. The most accurate description of the site of the chemoreceptor trigger zone for emesis which can be given at present is based solely on topographical relationships of the lesion to known medullary nuclei. In a composite appraisal of all specimens, the area of ablation was observed histologically to be a very small superficial pocket situated in the dorsal region of the ala cinerea. Only minimal damage was found in the vagal nuclei. To the authors' knowledge, the region herein localized has not previously been analyzed morphologically. This study is now in progress and the findings will be published in a separate paper.

Discussion. Investigations on the mechanism of digitalis-induced vomiting have resulted in a highly controversial literature. For a good survey of the subject, the reader is referred to Dresbach(7). This worker very painstakingly interrupted as far as possible every known visceral afferent pathway to the medulla oblongata without eliminating digitalis emesis. Although all recent indications have pointed to a central emetic action of digitalis, the exact site of this action has never been demonstrated. The isolated observation made by Koppanyi(3) that intravenous digitalis failed to induce emesis in two dogs with chronic lesions in the ala cinerea went unheeded despite its potential significance. Both of his animals had rather widespread but incomplete destruction of the dorsal vagal nuclei. His results were undoubtedly the consequence of diffuse damage which included the chemoreceptor trigger zone located in the dorsal region of the ala cinerea.

On the basis of our earlier work on the vomiting center(4,5) and the present experiments on the central site of action of digitalis, a new concept of the medullary regulation of vomiting has been evolved. According to this concept, drugs such as apomorphine and digitalis glycosides do not exert a direct action on the emetic center. Vomiting is initiated through reflex circuits, regardless of whether the receptor site is peripheral, as in the gastrointestinal mucosa, or central, as in the chemoreceptor trigger zone of the medulla. It is interesting that Hatcher and Weiss (cited by Hatcher)(8), after observing the sudden inhibition of vomiting by the scratch and defecation reflexes, concluded that vomiting is purely reflex in nature.

The present delimitation of the central locus of emetic action of the cardiac glycosides by no means excludes the possibility that reflex vomiting may be initiated at certain peripheral sites by these drugs. Indeed, the fact that a delayed emetic response to intravenous scillaren A was observed in one

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8. Hatcher, R. A., *Physiol. Rev.*, 1924, v4, 479.

successfully operated dog suggests that there may be a site of emetic action for the cardiac glycosides other than in the medulla.

As far as we are aware, the trigger zone for emesis in the medulla oblongata represents the first such discrete and specialized chemoreceptor area clearly demonstrated to reside within the central nervous system. Speculation on this discovery suggests certain broad implications for chemoreceptor physiology in general, and the need for re-evaluation of "centrally" acting drugs in particular.

Summary. Experiments on dogs have revealed the existence of a chemoreceptor trigger zone for emesis which is quite distinct from the vomiting center. This zone is a bilateral structure situated at the surface of the medulla oblongata in the dorsal region of the ala cinerea. Destruction of the emetic chemoreceptor zone results in animals which are permanently refractory to apomorphine given by vein, but it does not impair the vomiting response to orally administered copper sulfate. Eight out of 9 such dogs failed to vomit when tested intravenously with known emetic doses of the cardiac glycosides, lanatoside C, scillaren A and ouabain. Only one vomiting response was elicited; in this case, the latent period was greatly prolonged, a fact which suggests the existence of another site of emetic action of digitalis glycosides. The significance of a central chemoreceptor trigger zone for the emetic action of digitalis is discussed.

Received January 16, 1951. P.S.E.B.M., 1951, v76.

Effect of Renal Decapsulation on Hypertension Induced by Single Episode of Acute Choline Deficiency.* (18483)

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When weanling rats are fed a properly devised choline deficient ration, within one week they develop renal lesions characterized grossly by subcapsular bleeding so that the syndrome has been called simply "hemorrhagic kidneys"(1). If at this time the rats are transferred to stock rations, many of them recover. However, over the course of several months these animals develop systemic arterial hypertension of varying degree(2,3). Since the earliest descriptions of the "hemorrhagic kidney syndrome" noted that the kidneys of those animals which survived the acute episode appeared "frosted"(1), presumably denoting a fibrotic capsule, it seemed possible that the pathogenesis of hypertension in these rats might be similar to that in animals in which hypertension has been induced by coating the kidney with silk, cellophane, acrylic resin or other plastic material. This would seem particularly likely if such a fibrotic capsule is unable to grow at a rate commensurate with that of the renal parenchyma. The present study was designed to test this hypothesis.

Experimental. The experimental animals were males of the Vanderbilt strain(4). When they had attained a weight of 40 g they were housed in individual wirebottomed cages and fed the choline deficient diet(3) *ad libitum.* After 6 days, each rat was given a single dose of 10 mg of choline chloride by pipette and returned to a commercial stock

* This study was supported by a grant-in-aid from the Life Insurance Medical Research Fund. The authors' thanks are due to Merck and Co., Rahway, N. J., and to the Lederle Laboratories, Pearl River, N. Y., for a generous supply of crystalline vitamins and to Mrs. Eunice Matthews and Miss Ludie Avery for technical assistance.

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2. Best, C. H., and Hartroft, W. S., *Fed. Proc.,* 1949, v8, 610.
3. Handler, P., and Bernheim, F., *Am. J. Physiol.,* 1950, v162, 189.
4. Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Physiol.,* 1938, v34, 352.

ration.† Of 58 rats so treated in the present study, 9 died of the acute renal lesions. One week later, both renal capsules were stripped and removed from 13 of the surviving rats (Group A) under sodium pentobarbital anesthesia and the kidneys replaced in their normal position. Care was taken not to rupture the peritoneal wall.

A second group of 12 choline deficient animals (B) was allowed to develop hypertension and, 12 weeks after the acute renal episode, their kidneys were decapsulated in similar fashion. A third group of choline deficient animals (C) served as controls. As further controls another group of 12 rats (D) were fed stock ration throughout, the choline deficient period being omitted, but renal decapsulation was performed when they weighed 125 g. Four weeks after the initial choline deficient period, all rats attained a size sufficient to permit satisfactory estimations of systolic blood pressure with a modified version of the end-point device of Skeggs and Leonards (5) similar to that of Chittum, Hill and Grimson(6) Weekly blood pressure estimations were made, thereafter, and the results are shown in Fig. 1. The values plotted represent the simple arithmetic mean of all animals in each group.

As found previously, virtually all rats subjected to the episode of choline deficiency became hypertensive in varying degree although few rats showed pressures exceeding 160 mm after 16 weeks. This may have been due merely to the relatively brief duration of these experiments. Decapsulation immediately following choline deficiency (Group A), resulted in an actual elevation in pressure during the first few weeks of the experiment. However, as seen in Fig. 1, this transient hypertension was independent of the choline deficiency experience since it was also observed in Group D. The effect was relatively short lived and after 10 weeks the blood pressures of both groups subsided to normal

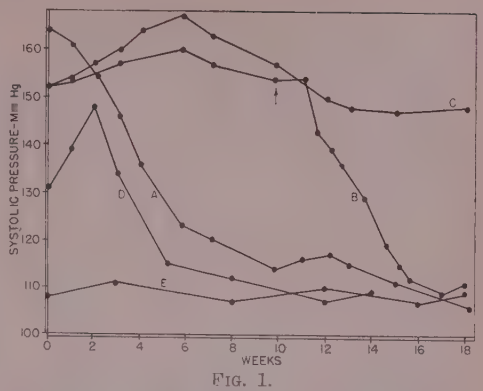

FIG. 1.

Effect of renal decapsulation on hypertension due to hemorrhagic kidney syndrome. Groups A, B, and C were choline deficient for a one week period, 3 weeks before 0 time, then were maintained on chow. The kidneys of group A were decapsulated one week after the choline deficiency episode, while those of group B were decapsulated at time indicated by the arrow. Group C was not decapsulated. Group D was not choline deficient, but was decapsulated one week before 0 time. Group E were normal controls.

levels and remained there for the duration of the experiment. As seen from the behavior of group B, renal decapsulation not only prevented the hypertension which is ordinarily observed subsequent to choline deficiency, but also abolished such hypertension after it had been established.

It had previously been shown that the hypertension of partially nephrectomized rats disappears when they are fed a low protein diet(7) and that the hypertension can be restored by the administration of a dose of ACTH which is without effect on the blood pressure of normal rats(8). It seemed of interest, therefore, to determine the effect of ACTH on the blood pressure of the decapsulated rats described above. In Fig. 2 is shown the effect of single, daily, intraperitoneal doses of 1 mg of ACTH‡ to rats of groups A, C, D, and E during the 14th week

† Ralston-Purina Co., St. Louis, Mo.

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8. Handler, P., and Bernheim, F., *Am. J. Physiol.*, 1950, v162, 368.

‡ Obtained from Armour and Co., Chicago, Ill., through the courtesy of Dr. J. D. Fisher. This preparation was from lot 60-61 and was stated to have a biological potency of 100% of standard La-I-A.

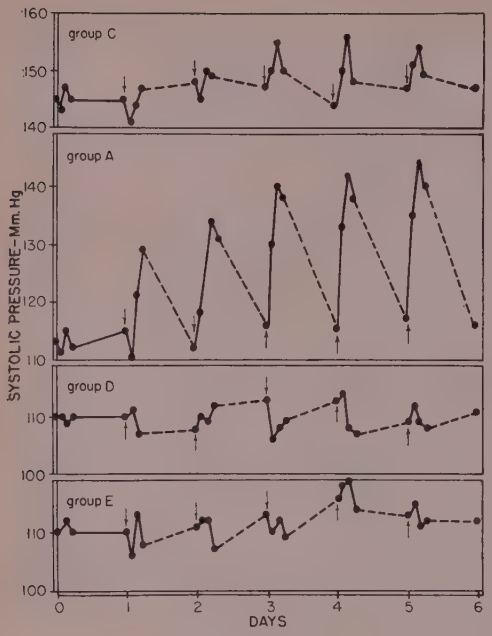

FIG. 2.

Effect of ACTH on blood pressure of renal decapsulated rats. For explanation of groups see text and Fig. 1. ACTH (1 mg) was given at times indicated by the arrows.

of the experiment. While this treatment did not affect the normal rats of groups D and E or the hypertensive rats of group C, it effectively induced hypertension in the erstwhile choline deficient group in which hypertension had been prevented by renal decapsulation.

At the termination of these experiments, the kidneys of 9 of the animals in group A and 6 each from groups C, D, and E were fixed in formaldehyde. These were examined histologically by Dr. R. H. Follis, Jr.§ of the Johns Hopkins School of Medicine who reported, "There are no changes in the parenchyma or vessels of any group which set them apart from the others. Group C does show thicker capsules than the other capsulated kidneys (groups D and E) while no capsules were apparent in group A. In no case did there appear to be evidence of an excess vascular supply."

Discussion. It is apparent from these data that renal decapsulation can both prevent and alleviate the hypertension ordinarily consequent upon a single episode of acute choline deficiency. No simple explanation is at hand to account for this relationship between the renal capsule and blood pressure. However, these findings are compatible with the hypothesis that the hypertension under consideration may be initiated by an inability of the renal capsule to grow normally after recovery from the "hemorrhagic kidney syndrome". After this work was completed there appeared the report of Danford, *et al.*,(16) which demonstrated that surgical removal of the silk capsule from the kidneys of rats in whom hypertension had been induced by this means resulted in a fall in blood pressure to normal values in 3 of 4 animals. Thus, the analogy appears quite complete. Hartroft(17) has found that renal decapsulation, performed *prior* to the development of 'hemorrhagic kidneys' does not prevent or alleviate this syndrome. As the rats did not survive the acute disease, this author could not observe the subsequent effect of decapsulation on blood pressure. In the present work, however, renal decapsulation was performed 1 and 12 weeks *after* the acute episode of 'hemorrhagic kidneys', yet was effective in interrupting the events ordinarily consequent upon this acute disease.

Renal decapsulation has also been found to alleviate the hypertension of dogs with artificially constricted renal arteries(9,10) although this procedure has not proved beneficial in human hypertensives(11-13). However, the concept involved in these latter studies was of a different nature. Previous

§ We are especially indebted to Dr. Follis for his kindness and cooperation.

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workers have been careful to cover the decapsulated kidney with omentum so as to develop a collateral renal circulation(14). In the present experiments, after decapsulation, the kidneys remained in their normal anatomical relationships. At autopsy the decapsulated kidneys were surrounded with the usual perirenal adipose tissue and there was no evidence of any collateral circulation. However, the decapsulated kidneys did present rather bizarre, lobulated forms and were invariably somewhat larger than the normal kidneys of corresponding animals, both choline deficient and non-choline deficient.

Whatever may be the role of the capsule in this situation, it cannot be the sole factor involved. As shown by the effects of ACTH, even after decapsulation, there remains considerable functional impairment. It has been found in this laboratory that ACTH, in this same dosage, will restore the hypertension of rats from which ¾ of their renal tissue has been removed and in whom hypertension has been prevented by feeding a low protein diet(8) and will also produce hypertension in bilaterally nephrectomized rats while they survive(15), yet is without effect on unilaterally nephrectomized rats. Thus, the renal decapsulated, normotensive post-choline deficient rats of the present study may be considered to have, in this sense, renal function comparable to that of an animal with somewhat less than one intact kidney. There may be an analogy between such rats and humans who develop hypertension under stress or 'tension' yet appear to have normal renal function by the usual criteria.

Summary. Bilateral renal decapsulation, performed one week after an acute episode of 'hemorrhagic kidneys' in choline deficient rats, prevents the development of hypertension in such animals and restores blood pressure to normal when the operation is performed in rats in which such hypertension is already established. Administration of ACTH to such renal decapsulated rats restores their hypertension but is essentially without effect on the pressures of normal rats, hypertensive rats, or renal decapsulated rats which never were choline deficient.

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Received November 28, 1950. P.S.E.B.M., 1951, v76.

A Microbiological Assay Method for Biotin. (18484)

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Microbiological methods for the determination of biotin have been employed in many laboratories using several microorganisms (*Lactobacillus casei, L. arabinosus, Clostridium butylicum, Leuconostoc mesenteroides, Rhizobium trifolii*)(1,2). Although 2 molds (*Neurospora crassa* wild and cholineless, and *Nematospora (Ashbyia) gossypii)* have been used as test organisms for biotin(3-5) they have not yet been generally used.

In the present paper a new method is described based on the biotin requirement of the filamentous fungus *Allescheria boydii*. This observation was first reported by Cury (6) and a more detailed study on vitamin nutrition related to biotin was published by Villela and Cury(7). It has been shown that *A. boydii* is a specific organism for biotin and that the growth responses are proportional to the amount of this vitamin in the medium.

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TABLE I. Basal Medium.

Dextrose (anhydrous)	30.0 g
Asparagine (biotin-free)	2.0 "
$MgSO_4 \cdot 7H_2O$	0.5 "
KH_2PO_4	1.5 "
Trace elements sol.*	1.0 ml
Distilled water to	1000 "
pH adjusted to 5 with 2 N NaOH	

* The stock solutions of trace elements consist of: 28.2 mg of H_3BO_3, 81.6 mg of $CuSO_4 \cdot 5H_2O$, 40.55 mg of $MnSO_4 \cdot 4H_2O$, 49.7 mg of $FeSO_4 \cdot 7H_2O$, 18.36 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 395.6 mg of $ZnSO_4 \cdot 7H_2O$ in 1000 ml of distilled water.

Therefore, we thought that it would be useful to work out an assay method for biotin.

Experimental. Organism. The test organism used is the strain of *Allescheria boydii* originally isolated by Boyd and Crutchfield from a mycetoma of yellow-white grains (strain N° 1699 of our culture collection). However, it was not possible to demonstrate experimentally any pathogenicity for animals with this organism. The other 4 strains investigated are unsuitable for the test since they grow without biotin. We believe that our strain N° 1699 is a natural mutant which has lost its capacity for synthesizing biotin. Stock cultures of *A. boydii* are maintained at room temperature by monthly transfers in a medium consisting of 3% dextrose, 1% peptone (Difco), 1% malt extract (Difco), and 1.5% agar.

Basal medium. The composition of the basal medium is shown in Table I.

Procedure. Assays are carried out in 50 ml Pyrex Erlenmeyer flasks cleaned with sulfuric acid-dichromate solution, thoroughly washed with tap water and rinsed with distilled water. The medium is made in double concentration and 5 ml distributed to each flask. After the convenient volume (1 to 5 ml) of the biotin standard solution or the extract to be tested are added the volume is completed to 10 ml with distilled water. The flasks are plugged with white cotton and autoclaved for 15 minutes at 120°C. In order to establish the curve for assay the stock biotin solution (50 μg per ml) is diluted to suitable concentrations. Aliquots of these diluted solutions are added to the flasks in duplicate in order to obtain 1 to 100 mμg of biotin per flask. A typical curve is shown in Fig. 1. Samples for assay are added in other flasks in volumes estimated to contain between 1 to 100 mμg of biotin. Volumes up to 5 ml may be used. The spore suspension for the inoculum is prepared by adding 10 ml of the basal medium to a culture and scraping the surface of the culture slightly with a platinum loop. A drop of the suspension is inoculated directly into the medium in each flask. The flasks are rotated to obtain a uniform mixture and incubated at room temperature for 8 days. They are then autoclaved for 15 minutes at 115°C. Then the pads are removed, washed thoroughly with distilled water, pressed out on filter paper, rolled in pellets, and dried at 110°C for 2 hours. After being cooled in a desiccator, they are weighed on an analytical balance. A reference curve with pure biotin should be established for each day's run. Controls (basal medium alone) are run parallel in all assays. The biotin content of the test substance may be determined by comparing the growth response of the unknown with that obtained with pure biotin. The average values encountered at 2 or more levels is used. Values taken below 1 and above 100 mμg should not be considered.

Reliability of method. The reliability of

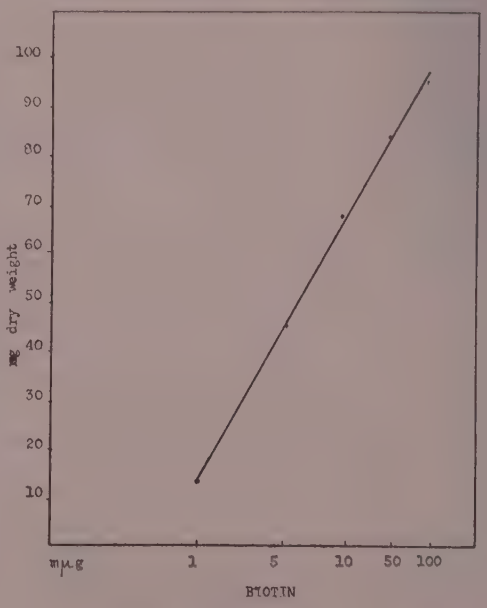

FIG. 1.
Growth response curve of *A. boydii* to biotin.

TABLE II. Reproducibility of Results with Known Amounts of Biotin.

Biotin, μg/10 ml	Dry wt (mg)										Mean and σ
.001	12.8	12.3	12.8	13.4	13.0	13.4	13.0	12.8	12.4	13.0	12.9 ± 0.36
.01	64.8	69.0	63.8	69.8	63.0	69.8	65.9	69.4	69.9	65.2	67.0 ± 2.88
.1	83.9	86.8	81.0	83.0	89.6	85.2	84.3	83.0	84.8	84.6	84.6 ± 0.74
.0	.0	.0	.0	—	—	—	—	—	—	—	—

$$\sigma = \text{Standard deviation} = \sqrt{\frac{\Sigma\,(x-m)^2}{n-1}}.$$

the method is indicated by good reproducibility of the determinations with pure biotin. As shown in Table II the reproducibility of the data are acceptable.

Preparation of samples for assay. For complete extraction of biotin from foods and tissues and liberation of biotin from the bound-biotin complexes the ground or blended samples are autoclaved in a convenient volume of 6 N HCl(1). After cooling the extracts are adjusted to pH 5.0 with N sodium hydroxide solution.

Specificity. The specificity of biotin for *A. boydii* has been studied(7). It has been shown that the biotin analogs (O-heterobiotin and dl-desthiobiotin) are able to replace biotin partially in the growth of *A. boydii*. When associated with biotin these analogs do not show an additive response. Cumulative effects are, however, observed only with amounts of 50 mμg or more of these analogs. Since these synthetic analogs are not found appreciably in natural products they will not interfere in the assays. It was also observed that nicotinic acid has some stimulative effect when associated with biotin. On the other hand, pyridoxin has a depressive effect on growth. This depressive action is neutralized by nicotinic acid. It is possible to avoid the interference of these vitamins by the use of selective adsorbents. Cysteine, glutathione, pimelic acid, aspartic acid, oleic acid and "Tween 80" are not effective in promoting the growth and do not interfere when associated with biotin. Biocytin, a naturally occurring complex of biotin recently isolated from yeast by Wright and coworkers(8), is effective in promoting the growth of *A. boydii* (9) as well as of *Saccharomyces cerevisiae* (10) although inactive for *Lactobacillus arabinosus*(8).

TABLE III. Assay Values and Recovery Experiments.

Material assayed	Biotin added (mμg/10 ml)	Biotin found (mμg/g sample)	Recovery (%)
Rat liver	0	.75	
	.17	.93	101
Yeast extr.	0	.67	
	.33	1.06	106
Casein (vit. free)	0	0	—
	.25	.25	100

Biotin assays and recoveries. Table III contains the assay values and recoveries of added biotin for 3 biological materials (rat liver, yeast extract, and purified casein). There are good recoveries of the added vitamin and the assay values given agree favorably with those reported in the literature.

Summary. A microbiological method for the quantitative determination of biotin is described. *Allescheria boydii* 1699 is used as test organism. The reliability of the method is indicated by the reproducibility of assays and good recoveries of the biotin added to various samples. The method is sensitive, the assay range being 1 to 100 mμg of biotin.

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Received November 21, 1950. P.S.E.B.M., 1951, v76.

Production of Experimental Uremia by Sodium Tetrathionate.* (18485)

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Gilman *et al.*(1) indicated that *in vivo* tetrathionate causes proximal renal tubular necrosis, uremia, oliguria or anuria, cast laden urine, and, that with doses of 500 mg/kilo of body weight or less, significant pathology is limited to the kidney. This suggests the use of tetrathionate for producing experimental uremia with fewer non-uremic complications than occur following use of other nephrotoxins, and is the concern of this paper.

Methods and materials. Six percent sodium tetrathionate† ($Na_2S_4O_6 \cdot 2H_2O$) solution was administered intravenously to mongrel dogs in doses of 100-500 mg/kilo of body weight. Morphine sulfate sedation was used when needed. Urines were collected daily, tested for reducing substances, specific gravity (SpG), examined for casts, and the volume noted, during control and uremic periods. Blood non-protein nitrogen (NPN) was determined daily by the Folin-Wu method(2). Carbon dioxide combining power (CO_2CP) was followed in one dog by the Van Slyke method(3). All dogs were sacrificed by intravenous pentobarbital and autopsied. Tissues taken for pathological study were fixed in formaldehyde and Zenker's solution.

Controls. To determine the presence of early, short-lived pathological changes, 3 dogs were given 450 mg of sodium tetrathionate/kilo of body weight, and one dog was sacrificed on the first, second, and third day respectively. Because reducing substances appeared in the urine following tetrathionate administration, fasting blood sugars were determined by the Folin-Wu method(4) in one dog for 3 consecutive days when the urine contained reducing substances. One normal dog was sacrificed to detect pathological changes resulting from pentobarbital administration.

Results. Uremia. Uremia of severity approximately proportional to the dosage of sodium tetrathionate resulted. Some animals responded to lower doses with a more severe uremia than did others to higher doses. Those receiving lower doses usually developed a reversible uremia (Fig. 1). Weight loss due to anorexia and dehydration was evident. Upper respiratory infections contracted during the uremia increased its severity. The CO_2CP fell from 65 vols % to 42 vols % in one animal upon which this determination was performed daily. Convulsions and vomiting occurred in those with severe uremia. One dog, after surviving an upper respiratory infection contracted during the course of its uremia, became chronically uremic, with an NPN which, at the time of writing, has varied from 75-120 mg % for more than 13 months.

Urine. Animals became anuric or oliguric following higher doses of tetrathionate. Urine which appeared was milky, opalescent, and greenish-yellow. It contained casts of all varieties, red, white, and epithelial cells, and reducing substances. The SpG fell to 1007-1011. Control pre-tetrathionate urines were normal.

Blood sugar. On the first 3 post-tetrathionate days, the blood sugar of one dog was 83, 93 and 100 mg % respectively when urines were 4, 4, and 3 plus respectively for reducing substances.

Pathological findings. Gross lesions. The

* The author wishes to acknowledge his indebtedness to Mr. Ronald Surbaugh for his assistance in handling the animals used in these experiments, to Messrs. Jack Handel, Eugene Leibsohn, and Michael West for technical assistance, and to Miss Sarah Cerza for preparing the tissues for pathological study.

† Supplied through the courtesy of Dr. Irwin C. Winter of G. D. Searle and Co., Chicago, Ill.

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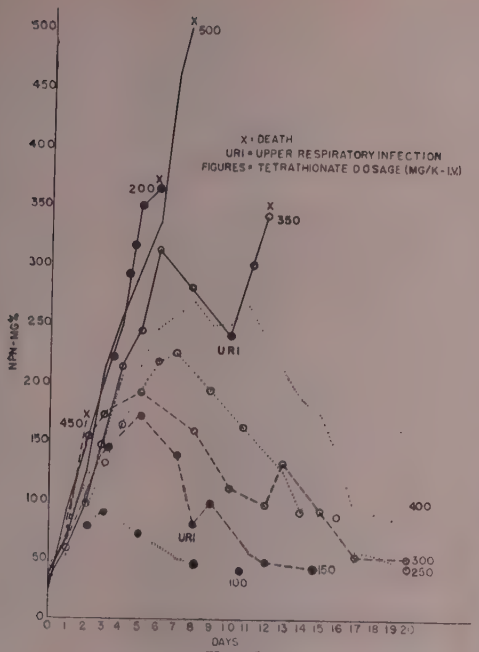

FIG. 1.

Blood NPN following intravenous administration of sodium tetrathionate. Each curve represents a different dog.

gross findings were passive congestion of all viscera, especially the liver and spleen, pulmonary edema and areas of atelectasis. Intussusception with submucosal hemorrhages and necrosis occurred in one animal.

Microscopic lesions.‡ The heart, lungs, liver, spleen, pancreas, adrenals and intestines showed no lesions not attributable to the terminal state, fatal pentobarbital intoxication, or uremia. The kidneys (Fig. 2 and 3) showed some enlargement of the glomeruli due to congestion and/or increased cellularity of the capillary loops, with proteinaceous fluid in a few Bowman's spaces. The majority of glomeruli were normal. The predominant renal lesion was coagulation necrosis with brightly eosinophilic staining cytoplasm and indistinguishable lumen spaces of the proximal convoluted tubules. Calcium deposition in necrotic epithelium was seen in older lesions (Fig. 3). Granularity, karyolysis, fraying, loss of brush border and vacuolar degeneration were present throughout all sections. Pink staining material in the lumina and sometimes only cloudy swelling were observed in milder cases. The lesions of the distal convoluted tubules resembled those of the proximal convoluted tubules, but their severity and extent were not as great. The collecting tubules showed hydropic vacuolization of their upper segments and pink staining material in some lumina. The interstitial tissue and vessels were moderately congested in a few cases.

The significant findings in the acute control animals receiving tetrathionate were confined to the kidneys (Fig. 2) and resembled

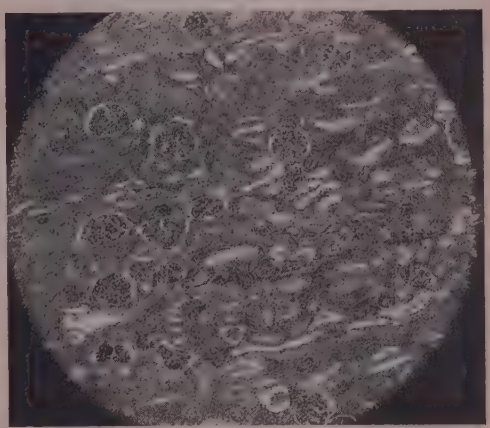

FIG. 2.

Acute renal lesions one day after 450 mg/kilo of sodium tetrathionate were administered intrav.

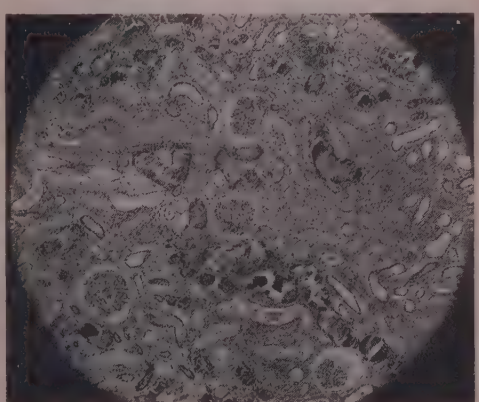

FIG. 3.

Renal lesions 13 days after 363 mg/kilo of sodium tetrathionate were administered intravenously. Note calcification of necrotic tubules.

‡ The author is indebted to Dr. M. Goldenberg of the Pathology Dept., The Chicago Medical School, Chicago, Ill. for these studies.

the findings described above. The pentobarbital control showed slight cloudy swelling of the kidney with casts in many of the loops of Henle. Other organs were normal or showed varying degrees of passive congestion.

Discussion. Surgical methods produce a uremia which is complicated by the dangerous and uncertain post-surgical state. Uremia produced chemically is often complicated by toxic reactions and uncertainty of results. The use of sodium tetrathionate as a nephrotoxin appears to overcome these disadvantages. The cause of the specific susceptibility of the tubule cells to the action of tetrathionate is not yet known. Gilman *et al.*(1) have discussed the possible mode of action of tetrathionate. The fact that the specific lesions produced by tetrathionate are limited to the kidney allows the production of experimental uremia without complications resulting from the technic used. The development of chronic uremia in one dog may have resulted from the contraction of an upper respiratory infection at a time when renal tubular proteins were circulating in the blood stream, with resultant formation of autoantigens and the subsequent development of auto-antibodies specific for the renal tubule.

Although one laboratory procedure, such as an NPN determination, cannot indicate the complete functional status of an organ as complex as the kidney, it can, when performed daily indicate the renal trend. Fig. 1 shows that increasing the dose of sodium tetrathionate from 100-500 mg/kilo of body weight increases the duration and severity of the uremia as indicated by the NPN and incidence of death. Those succumbing to lower doses were older animals; those surviving higher doses were much younger. This is in accordance with the findings of MacNider (5) who demonstrated that older dogs are more susceptible to nephrotoxins than younger ones.

The appearance of urinary reducing substances following tetrathionate administration required the ruling out of a hyperglycemia. In the control animal, fasting blood sugars were at low normal levels when fresh urines gave a 3 or 4 plus reaction with Benedict's solution, indicating that the reducing substances in the urine were due to tubular lesions and not a hyperglycemia.

Summary and conclusions. Uremia was produced in dogs by intravenous administration of varying doses of sodium tetrathionate. The findings of Gilman *et al.*(1) cited above were confirmed. In addition, it was shown that renal lesions resulting from tetrathionate administration, heretofore not described in detail, involve the distal convoluted and collecting, as well as the previously mentioned (1) proximal renal tubules. The resulting uremia may be rapidly fatal or continue 20 or more days, and is accompanied by non-hyperglycemic urinary reducing substances and lowered CO_2CP and SpG of the urine. The mechanism of chronicity following uremia complicated by an upper respiratory infection is briefly discussed. The specific action of tetrathionate on the kidney and the freedom from non-uremic complications following its use demonstrates its efficacy for producing experimental uremia.

Received September 11, 1950. P.S.E.B.M., 1951, v76.

Reversible Inhibition of Growth of *Microsporum audouini* with Neopyrithiamine.* (18486)

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Recent studies of the nutritional requirements of certain fungi have definitely established the essential nature of certain metabolites for growth and formation of spores. It has been shown that certain fungi are unable to synthesize these essential metabolites

TABLE I. Activity of Neopyrithiamine and Neopyrithiamine + Thiamine on Growth of *Microsporum audouini* and *Microsporum canis* from Infected Hairs.

μg per ml		*Microsporum audouini* No. 1	*Microsporum audouini* No. 2	*Microsporum canis*
Neopyrithiamine	Thiamine			
Control		++++	++++	++++
5		+ m	0	++
3		+ m	+ m	++
1		+ m	+ m	++
0.5		+ m	+ m	++++
3	.3	++++	++++	++++
3	.1	++++	++++	++++
3	.03	++++	++++	++++
3	.01	++	++++	++++

m = microscopic.

(amino acids, vitamins and so forth) and these deficient organisms will not grow unless the medium contains all factors essential for growth. Moreover, even in the presence of the essential factors, growth is prevented when the medium also contains compounds known as metabolic antagonists, which specifically interfere with the utilization of these factors by the organism(1). Unfortunately, little information is available regarding the factors essential for growth of pathogenic fungi attacking human beings. Conant(2) in his study of the microspora noted that *Microsporum canis* grew profusely on polished rice grains but that *Microsporum audouini* grew poorly and did not produce aerial growth or macroconidia. Further observations by Benedek(3), Hazen(4), de Arêa Leão and Cury(5) and Walker(6) suggested that *Microsporum audouini* was a deficient organism.

The following report demonstrates the inhibition of growth of *Microsporum audouini* with neopyrithiamine, a metabolic antagonist of thiamine. Thiamine, therefore, appears to be essential for growth of this organism.

Method. One stock strain of *Microsporum audouini* and the spores on fluorescent hairs from 3 patients, subsequently proved to be 2 strains of *Microsporum audouini* and 1 strain of *Microsporum canis*, were tested for their ability to grow on Sabouraud's dextrose agar (pH 6.8) which contained various concentrations of neopyrithiamine or of neopyrithiamine and thiamine. The stock medium used for testing was 20% more concentrated than the standard stock medium so that after addition of the reagent solutions, standard concentrations of nutrients would result. Serial dilutions of the reagents were prepared by placing the proper aliquot parts of reagents in Petri dishes and mixing them with 8 ml of the medium. The volume was kept constant at 10 ml by the addition of the proper amount of sterile distilled water. The stock strain of *Microsporum audouini* was heavily inoculated onto the plates as 0.2 ml of a ground suspension prepared from a week-old culture growing on Sabouraud's dextrose agar. The two strains of *Microsporum audouini* and one strain of *Microsporum canis* on fluorescent hairs were tested by placing several infected hairs on duplicate plates. The hairs were examined microscopically after they had been placed on the agar to determine whether or not spores were present. The plates were incubated at room temperature and examined periodically for growth for one month. Hairs were transplanted to Sabouraud's dextrose agar from one plate of the duplicate sets which did not show macroscopic growth in 14 days

* We are indebted to Dr. H. J. Robinson of Merck and Co., Inc., for the neopyrithiamine used in this study.

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TABLE II. Activity of Neopyrithiamine and Neopyrithiamine + Thiamine on Growth of a Stock Strain of *Microsporum audouini* on Solid and Liquid Media.

μg per ml		Growth	
Neopyrithiamine	Thiamine	Liquid medium	Solid medium
Control		++++	++++
.8		±	0
.4		±	+ m
.2		±	++
.8	.8	++++	++++
.8	.08	++++	++++
.8	.008	++++	++++

m = microscopic.

(Table I). The stock strain also was tested in liquid Sabouraud's dextrose medium. Dilutions of the test reagents were prepared in the same manner as for the Petri plates but in 28 by 100 mm tubes with a screw cap, which remained loose to allow aeration of the tube.

Results. The stock strain on solid medium did not show growth which could be detected either macroscopically or microscopically at a concentration of 0.8 μg per ml of neopyrithiamine (Table II). Growth was visible only microscopically at a concentration of 0.4 μg per ml of neopyrithiamine, but, although filaments began to develop, the amount of growth never became visible macroscopically even after a month of incubation. Growth which could be seen easily macroscopically was observed at a concentration of 0.2 μg per ml of neopyrithiamine but the amount of growth did not equal that on the control plate or on plates to which thiamine was added. This inhibition of growth by neopyrithiamine could be reversed by the addition of thiamine to the medium. Addition of as little as 0.008 μg per ml of thiamine could overcome the effects of 0.8 μg per ml of neopyrithiamine.

Neopyrithiamine was not as effective as an inhibitor in liquid medium as on the agar plates. At 0.8 μg per ml of neopyrithiamine, visible growth was evident after 4 days' incubation but growth did not continue as in the control tubes. After one month's incubation the medium in the control tubes was completely filled with mycelium and was covered over by a heavy pellicle. Tubes containing neopyrithiamine had no more growth after one month of incubation than was observed after 4 days. Neither the rate nor the amount of growth differed between control tubes and those containing 0.8 μg of neopyrithiamine and 0.8 μg per ml of added thiamine.

Results of planting hairs infected with *Microsporum audouini* or *Microsporum canis* on Sabouraud's dextrose agar containing various concentrations of neopyrithiamine or neopyrithiamine plus thiamine are recorded in Table II. It appears that higher concentrations of neopyrithiamine are required to inhibit the growth of *Microsporum audouini* from infected hairs than in pure culture transplants. Strain No. 1 of *Microsporum audouini* is less sensitive than strain No. 2 to the inhibitory effect of neopyrithiamine but also requires smaller amounts of thiamine to overcome the inhibition. Growth of *Microsporum canis* is somewhat retarded at a concentration of 1 μg per ml of neopyrithiamine but macroscopic colonies are easily visible in ten days and growth continues with incubation of the plates. Except for strain No. 2 originally planted on medium containing 5 μg per ml of neopyrithiamine, all hairs transplanted to Sabouraud's dextrose agar grew out in 4 days. After one week of incubation the hairs were removed from the one transplant which did not grow and again examined microscopically. The shafts were covered with the typical spore mosaic but germination was not evident.

Comment. Several vitamins have been reported as essential for the growth of *Microsporum audouini*(5). The inability of Hazen (4) to increase growth by the addition of thiamine and pyridoxine to a medium of honey agar does not eliminate these vitamins from the essential list. The quantity of factors producing growth which were present in the honey was not known. The results of Walker(6) show the requirement of these factors for some strains. The ability of neopyrithiamine to inhibit the growth of pure cultures of *Microsporum audouini* indicates a requirement for thiamine. This organism either cannot synthesize the vitamin or it produces thiamine so slowly that growth is hindered. The ability of the organism to in-

itiate but not to continue growth may be owing to the preformed thiamine contained within the organism at the time of transfer. Growth ceases or becomes extremely slow after this supply is exhausted. The growth of *Microsporum audouini* from infected hairs in concentrations of neopyrithiamine higher than that which completely inhibits pure culture transplants may be brought about by higher concentrations of thiamine present on the hair.

Unfortunately Sabouraud's dextrose agar is not devoid of thiamine. Stokes and coworkers(7) have found 0.11 to 0.28 μg of thiamine per gram of peptone. Assuming that the peptone used in this study contained the lower concentration of thiamine, each milliliter of medium would already contain 0.001 μg of thiamine. It seems reasonable to assume that on a purely synthetic medium entirely lacking in thiamine, still lower concentrations of neopyrithiamine would inhibit growth. An attempt has not been made to calculate the inhibition index from these data as the exact concentration of thiamine was not known. Experiments are being carried out on a synthetic medium to determine the index. It appears, with one exception, that the concentrations of neopyrithiamine employed did not kill all the *Microsporum audouini* during 14 days' contact. Strain No. 2 originally planted on medium containing 5 μg per ml indicates, however, that in higher concentrations neopyrithiamine may be fungicidal in the same period.

Summary. The ability of neopyrithiamine to inhibit growth of *Microsporum audouini in vitro* in very low concentrations suggests its use in cases of tinea capitis caused by this fungus. The application of neopyrithiamine alone or in combination with other agents or vitamin antagonists now being studied may prove helpful in combating these infections.

Received December 6, 1950. P.S.E.B.M., 1951, v76.

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Vitamin B_{12}, a Factor in Prevention of Hydrocephalus in Infant Rats.* (18487)

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During a study of the adequacy of a synthetic-type, casein diet for reproduction and lactation in the rat, Richardson and Hogan (1) observed that about 2% of the offspring were hydrocephalic. They ascribed this abnormality to a nutritional deficiency and showed that it could be prevented by an aqueous extract of liver. O'Dell, Whitley and Hogan(2) showed that the addition of folic acid to a diet containing casein as the source of protein usually, but not always, prevented the abnormality. More recently Hogan, O'Dell and Whitley(3) observed hydrocephalus in about 20 percent of the offspring when a folic acid inhibitor, crude methylpteroylglutamic acid (Methyl PGA), was added to a diet that contained soybean oil meal as the source of protein. The type of diet consumed during the pre-experimental period had a marked effect on the incidence of hydrocephalus among the first few litters produced by each dam, a fact which suggested that a protective factor may be stored in the

* Contribution from the Department of Agricultural Chemistry, Mo. Agric. Exp. Station, Journal Series No. 1243. This investigation was supported in part by a grant from the U. S. Public Health Service.

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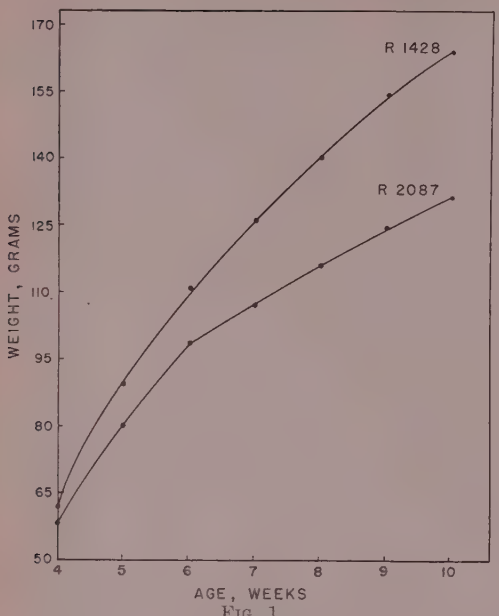

FIG. 1.

Age-weight curve of female rats. The experimental rats were transferred at 4 wk of age from the stock colony diet, R1428, to the depletion diet, R2087, and their litter mate controls were continued on R1428. There were 22 animals on each diet.

body of the mother rat. The purpose of this communication is to show the value of vit. B_{12} in the prevention of hydrocephalus.

Experimental. (a) Depletion of the protective factor. After it was observed that the pre-experimental diet has a profound effect on the incidence of hydrocephalus, all female rats used subsequently were depleted by maintaining them from weaning to maturity on the basal ration, No. 2087(3), which has the following percentage composition: Soybean oil meal 70, cerelose 22, lard 4, and salt mixture(1) 4. All recognized vitamins were added to this diet except ascorbic acid, niacin, folic acid, inositol, and vit. B_{12}.† The adequacy of Ration 2087 for growth is shown in Fig. 1 which compares the growth of female rats, taken from the stock colony at 4 weeks of age and fed Ration 2087, with litter-mate controls fed the stock ration, No. 1428. It has been shown by data not presented here that when Ration 2087 is supplemented with a vit. B_{12} concentrate that supplies 2.2 μg/100 g ration, the rate of growth equals that obtained on the stock ration. Thus the basal ration is low in vit. B_{12} but does not show any other serious deficiency for growth of the rat.

(b) Addition of a vit. B_{12} concentrate to the diet. Since the basal ration is deficient in vit. B_{12} and since one factor important in the prevention of hydrocephalus is stored for relatively long periods in the animal body, it seemed probable that vit. B_{12} is important in the prevention of the abnormality. To test its value three groups of animals were used. Group I was maintained on the basal ration for a period of at least three months in order that the animals might be thoroughly depleted of the protective factor. The animals then received, during the experimental period, Ration 2087 to which was added 1, 2, or 4 mg of crude Methyl PGA per 100 g of ration. Group II was not depleted but received the same experimental diets as Group I. Group III was not depleted and received, in addition to the Methyl PGA, a vit. B_{12} supplement in the form of a concentrate‡ which supplied 2.2 μg per 100 g of diet. Since there was no difference in response to different levels of antagonist in any group, the results of the different levels are combined in each case to simplify presentation. The results are presented in Table I.

The incidence of hydrocephalus was 23% among the offspring from depleted dams and 15% from undepleted females. This difference was striking among first and second litters but after the third litter there was little difference. When the dams received vitamin B_{12} in the diet there was no hydrocephalus among the 1261 offspring observed although some individual females bore a total of seven litters during the experimental period. When vit. B_{12} was omitted a low percentage of the offspring was weaned, with most of the

† The folic acid was supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., and all other B-complex vitamins through the courtesy of Dr. D. F. Green, Merck and Co., Rahway, N. J.

‡ The vit. B_{12} concentrate was Merck's Animal Protein Factor Supplement which contained according to microbiological assay 12.5 mg per lb.

TABLE I. Relation of the Incidence of Hydrocephalus to the Vit. B_{12} Content of the Diet.

Additions to ration 2087	Litters born: Total	Litters born: With hydrocephalic young	No. born	% weaned at 4 wk*	Hydrocephalic offspring: No.	Hydrocephalic offspring: %
		Group I—Pre-experimental depletion				
Methyl PGA†	144(6)‡	77	949	17	222	23
		Group II—No pre-experimental depletion				
Methyl PGA	241(8)	96	1568	21	237	15
		Group III—No pre-experimental depletion				
Methyl PGA + B_{12} conc.	166(7)	0	1261	81	0	0

* The % weaned is based on the number born alive.

† Crude methylpteroylglutamic acid supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

‡ Numbers in parentheses indicate maximum number of litters produced by any one female during the experimental period.

mortalities occurring during the first 2 to 3 days of life. The young rats had gross symptoms similar to those described by Schultze(4). According to Schultze uremia was prevented by the subcutaneous injection of vitamin B_{12} in infant rats, but their weaning weights were not improved. Under our conditions the subcutaneous injection of vit. B_{12} did not decrease the rate of mortality among the offspring of B_{12}-depleted dams. Of 20 new-born rats injected with 0.1 microgram of vit. B_{12} according to the technic described by Schultze only 2 survived to 1 week of age and of 21 untreated litter-mate controls 4 survived. It seems that there is some defect at birth which is not readily corrected by administering vit. B_{12} at that time. The offspring of dams that received vit. B_{12} in the diet were vigorous and 81% of those born alive were weaned. In addition to the high mortality and hydrocephalus which occurred among the offspring, other abnormalities have been observed. These include occasional cases of spina bifida, cranium bifidum, edema, anophthalmia or microphthalmia, hare lip, cleft palate, and short lower mandible. The total or partial absence of the eyes as well as other eye abnormalities seems to be closely associated with the incidence of hydrocephalus.§ Yudkin(5) reported the occurrence of congenital anophthalmia in a family of albino rats but its cause was not determined.

(c) Parenteral administration of crystalline vit. B_{12}. The vit. B_{12} used in the feeding trial described above was in the form of a concentrate and there was thus the possibility that the activity was due to another substance in the concentrate. In order to test crystalline vit. B_{12} 5 female rats were selected that had produced at least 3 consecutive litters with hydrocephalic young. In these 3 litters there were 97 young born, 43% of which were hydrocephalic and only 16% of which survived to 4 weeks of age. The females then received parenterally 1 μg of crystalline vit. B_{12} (Cobione) each week beginning at least 2 weeks before conception and continuing throughout gestation. The results are shown in Table II. Each female produced 2 litters while receiving crystalline vit. B_{12}. No hydrocephalic offspring were observed and 75% of the young survived to 4 weeks of age. The hydrocephalus observed in these infant rats is congenital and is almost always detectable at birth. It seemed of interest therefore to determine at approximately what stage of embryonic development the damage to the brain occurs. For this study female rats which had consistently produced hydrocephalic offspring were injected parenterally with 1 microgram of vit. B_{12} at different stages of gestation. The results of these treatments are shown in Table II. One group of 7

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§ Mr. Fred Ransdell has undertaken a histological study of the eyes of hydrocephalic animals.

5. Yudkin, A. M., *Am. J. Ophthalmology*, 1927, v10, 341.

TABLE II. Effect of Parenterally Administered Vit. B_{12} on the Incidence of Hydrocephalus.

Pre-experimental period					Experimental period				
					Litters				
No. of females	No. of litters	No. born	% weaned	% hydrocephalic	Total	With hydrocephalus	No. born	% weaned	% hydrocephalic
Treatment: 1 μg vit. B_{12} weekly for 5 wk before parturition									
5	15	97	16	43	10	0	67	75	0
Treatment: 1 μg of vit. B_{12} on 7th day of gestation									
7	21	147	3	39	7	0	42	14	0
Treatment: 1 μg vit. B_{12} on 14th to 16th day of gestation									
5	15	126	21	37	5	4	28	14	50

TABLE III. Effect of Folic Acid on Incidence of Hydrocephalus When Fed in the Absence of Vitamin B_{12}.

Additions to ration 2087	Litters		No. born	% weaned	% hydrocephalic
	No.	With hydrocephalic young			
1 mg % methyl-PGA*					
5 mg % folic acid	7	7	30	17	40
0.5 mg % folic acid	20	10	155	11	28

* The females used in this group had previously produced 3 consecutive litters with hydrocephalic offspring.

females was treated on the 7th day of gestation and they produced 7 litters with a total of 42 young none of which was hydrocephalic. Thus it appears that 1 μg of B_{12} administered on the seventh day of gestation is sufficient to prevent hydrocephalus in at least the majority of cases, but it does not allow the production of viable offspring since only 14% were weaned. Five females were treated with 1 μg of vit. B_{12} on the fourteenth to sixteenth days of gestation and 4 out of 5 litters contained hydrocephalic young. Two females not included in the table were treated on the twelfth and thirteenth days respectively and their litters were free of hydrocephalus. The data available are insufficient to draw conclusions as to the exact time when vit. B_{12} must be administered to prevent the abnormality, but it is clear that the damage occurs after the seventh day of gestation and probably around the twelfth to fourteenth day.

(d) Addition of folic acid without vit. B_{12}. Folic acid is effective in decreasing the incidence of hydrocephalus when the females consume a diet that contains 30% of casein(2), but this protein is usually contaminated with a significant amount of vit. B_{12}. It seemed desirable to determine whether folic acid is still effective when the diet is practically devoid of vit. B_{12}. For this purpose folic acid was added to Ration 2087 and to a similar ration containing crude Methyl PGA and these rations were fed to female rats that had been depleted of vit. B_{12}. The results are shown in Table III. Hydrocephalus was not prevented in vit. B_{12} depleted dams by the addition of folic acid to the basal diet, and it made little difference in the incidence whether the antagonist was added or omitted. Hogan, *et al.*(3) reported that the incidence of hydrocephalus on the basal diet was low unless the folic acid antagonist was added. These observations were made on the offspring from the first 3 litters of females that had not been depleted. When these females were then fed the diet supplemented with the antagonist the incidence of hydrocephalus rose sharply, suggesting that the antagonist induced a folic acid deficiency. It is reasonable to believe that the antagonist did precipitate the occurrence of hydrocephalus but it is unlikely that a severe folic acid deficiency existed since Franklin, *et al.*(6) found the

6. Franklin, A. L., Stokstad, E. L. R., Belt, Margaret, and Jukes, T. H., *J. Biol. Chem.*, 1947, v169, 427.

ratio of antagonist to folic acid necessary to produce deficiency symptoms in rats fed succinylsulfathiazole to be 3000. According to microbiological assay, the basal ration contained at least 0.3 mg of folic acid per 100 g. The data in Table III show that if female rats are depleted of vit. B_{12} they produce litters with a high incidence of hydrocephalus, even though the basal diet is supplemented with folic acid.

Discussion. In our early work casein was the source of protein and the incidence of hydrocephalus was under 2%. When folic acid (O'Dell *et al.*)(2), was added to the diet the incidence of the abnormality was practically zero indicating that folic acid is an important preventive factor. The importance of folic acid was supported by the observation of Hogan *et al.*(3) that when female rats consumed a soybean oil meal diet the addition of a folic acid inhibitor precipitated the occurrence of hydrocephalus. However, the failure of folic acid to reverse the inhibition made it seem doubtful that folic acid was the only factor involved. According to microbiological assay casein usually contains more vit. B_{12} than does soybean oil meal, and this suggested that a deficiency of vit. B_{12} may be implicated in the abnormality. Our recent studies show that when this vitamin is administered to female rats on a soybean oil meal diet which contains low levels of Methyl PGA their offspring are not hydrocephalic. Since the level of inhibitor used is unlikely to induce a marked folic acid deficiency it is uncertain whether or not an adequate level of vit. B_{12} in the absence of folic acid will prevent hydrocephalus. It is certain, however, that folic acid, in the absence of vit. B_{12}, will not prevent the abnormality. Studies are now under way to determine whether both of these vitamins are required.

Summary. After female rats, on a diet that contained soybean oil meal as a source of protein, became depleted of the factor that prevents hydrocephalus, the incidence of the abnormality among the offspring was 28%. The addition of a vit. B_{12} concentrate to the diet or the injection of crystalline vit. B_{12} in the dams during the early stages of gestation prevented the abnormality in the young animals and increased their viability. Hydrocephalus was not prevented by folic acid alone, but it has not been determined whether vit. B_{12} alone is effective or whether both folic acid and vit. B_{12} are required.

Received January 15, 1951. P.S.E.B.M., 1951, v76.

Effect of Treatment with ACTH or Cortisone on Anatomy of the Brain.* (18488)

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Much evidence indicates that adrenocortical hormones exert important effects on the central nervous system. In rats sensitized by unilateral nephrectomy and feeding of high salt diets, Selye(1) induced inflammatory vascular lesions and edema in the brain by the administration of cortisone. In man, euphoria, psychotic reactions, restlessness, convulsions(2), and irregularities in the electroencephalogram(3) have been observed

* Supported (in part) by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

† Student Research Fellow of the University of Michigan Medical School.

‡ The authors with to express their appreciation to Dr. Elizabeth C. Crosby for her generous advice during the course of this study.

1. Selye, H., *Stress,* Acta, Inc., Montreal, 1950.
2. Rome, H. P., and Braceland, F. J., *Proc. Staff Meeting, Mayo Clinic,* 1950, v25, 495.

following treatment with cortisone or ACTH. Psychotic states have been correlated, also, with decreased adrenal responsivity to adrenocorticotropin(4). Although it has been suggested that the hypothalamus controls the secretion of ACTH by the anterior hypophysis, there is no agreement concerning the localization of this function in the hypothalamus. Hume(5) contended that the integrity of the anterior hypothalamus is essential for the hypophysis to secrete ACTH in response to stress. Harris and De Groot(6) found that lesions in the posterior region of the tuber cinereum and in the mammillary body abolished the lymphopenic response to stress in rabbits. Stimulation of these regions resulted in lymphopenia. On this basis they concluded that secretion of adrenocorticotropic hormone is under neural control via the hypothalamus and the hypophyseal portal vessels. If this hypothesis is correct it would be reasonable to expect that treatment with ACTH or cortisone would produce microscopic evidence of reduced secretory activity in the hypothalamus.

In this investigation we have studied the effect of administration of ACTH and cortisone on the portion of the brain extending from the olfactory bulbs to the region just caudal to the posterior commissure and the mammillary bodies, thus including the cerebral cortex, thalamus and hypothalamus.

Procedure. Four rats received ACTH by continuous subcutaneous injection for 21 days at a dosage of 6 mg per day. Since ACTH disappears rapidly from the blood stream(7), this method of administration is necessary in order to secure the maximal physiological effect of the hormone. A fifth rat received 1 mg of ACTH per day in 8 divided subcutaneous injections. Three rats received 10 mg of cortisone acetate (Merck) per day by intermittent injections for 10 days. For each rat receiving hormone a control was injected with the vehicle. Adult, male, Sprague-Dawley rats were used and at the beginning of the experiment the difference between the body weights of the treated and non-treated rats in each pair was less than 10 g. The control rats gained in weight during the experiment whereas those receiving ACTH lost, on an average, 35 g; and those receiving cortisone, 40 g. The ACTH was prepared by the method of Li, Evans and Simpson(8). The animals were fed by stomach tube according to the method of Reinecke, Ball and Samuels(9). The brains were fixed in Carnoy's fluid. Frontal serial sections were cut at 12 and 20 μ and stained with .5% toluidine blue. Insofar as possible, control and experimental sections were matched area for area and the slides stained in pairs. The nuclear masses identified and studied were for the hypothalamus those listed by Rioch, Wislocki, and O'Leary(10) and Gurdjian(11) and for the thalamus those listed by Gurdjian (11). In the cerebral cortex, the areas designated by Krieg's(12) numerical nomenclature were investigated. All of the above named areas in which changes are not described were normal.

Results. ACTH-treated rats. Consistent cytological changes were observed only in the hypothalamic paraventricular nucleus, these occurring in all 5 animals receiving the hormone. There was an over-all diminution in cytoplasmic basophilia throughout the paraventricular nucleus, the decrease in Nissl substance frequently being most marked in the perinuclear areas (Fig. 1-4). Some cells were affected more than others. In those affected most severely, the total cell volume was re-

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11. Gurdjian, F. S., *J. Comp. Neurol.*, 1926, v42, 1.

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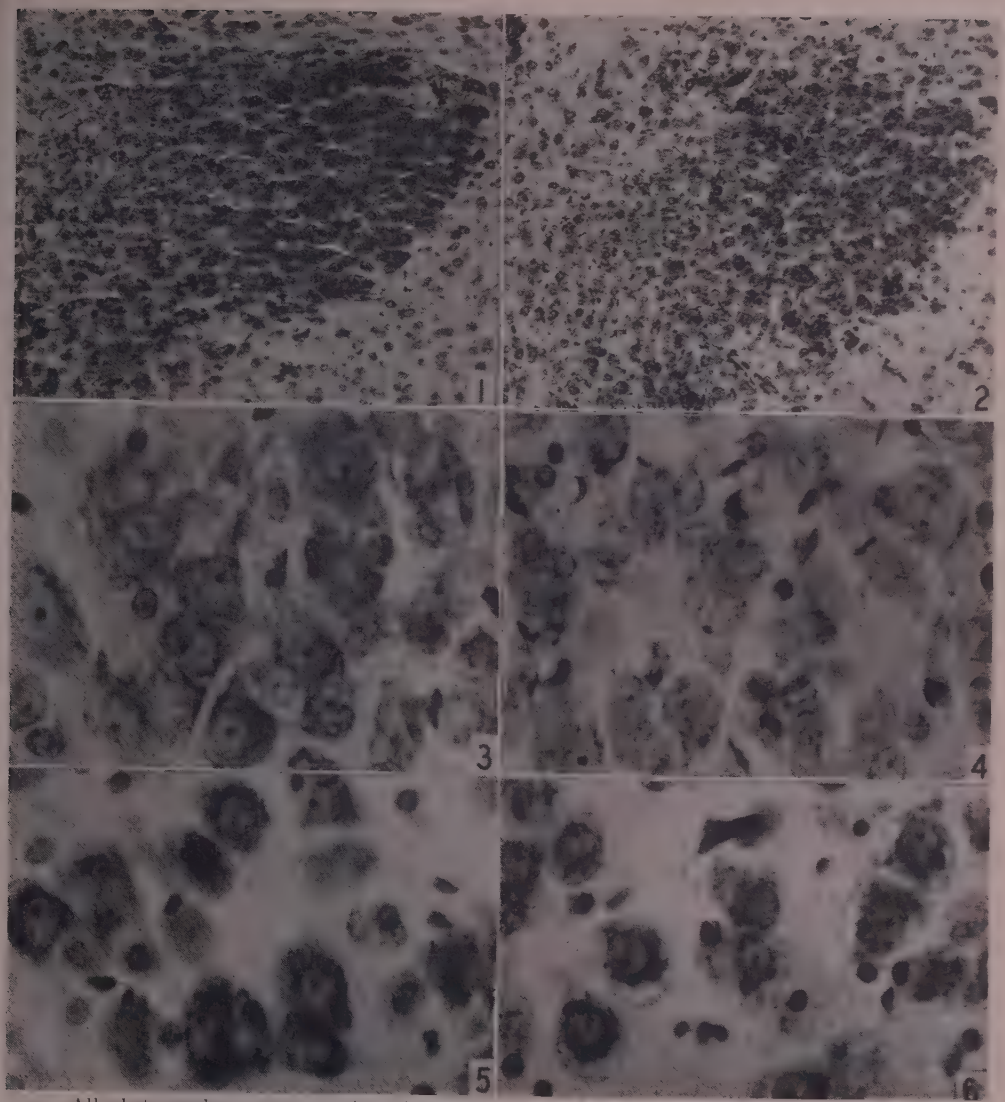

All photographs are preparations fixed in Carnoy's fluid and stained with Toluidine blue.

FIG. 1. Hypothalamic paraventricular nucleus of a control rat. ×125.

FIG. 2. Paraventricular nucleus of a rat treated with 6 mg of ACTH daily for 21 days by continuous injection. The amount of Nissl substance is reduced. ×125.

FIG. 3. Cells of the paraventricular nucleus of a control rat showing the distribution of Nissl material in the cytoplasm and large size of the nucleolus. ×680.

FIG. 4. Cells of the paraventricular nucleus of a rat treated as for Fig. 2. The cells, nuclei, and nucleoli are smaller and the cytoplasm is depleted of Nissl substance. ×680.

FIG. 5. Nissl material in the cells of the anterodorsal nucleus of the thalamus of a control rat. ×575.

FIG. 6. Chromatolysis and vacuolation of the cells in the anterodorsal nucleus of the thalamus after treatment with cortisone. ×575.

duced, the nucleus was smaller, less chromatic and contained a smaller nucleolus. In the anterior hypothalamic area, there was some suggestion that the number of cells containing Nissl material was reduced in the treated rats.

In 2 of the 4 rats treated with the 6 mg

dose of ACTH, the cells of the *N. reuniens* and *N. rhomboidalis* of the thalamus were swollen and distorted with some chromatolysis and vacuolation of the cytoplasm. One rat which received 6 mg of the hormone daily showed swelling and chromatolysis of the large ganglion cells in the stratum ganglionare throughout most of the cerebral neocortex (Krieg's(12) areas 4, 6, 8, 10, 24, 1, 2, 2a, 3, 39, 40, 17, and 18a).

Cortisone-treated rats. These animals were in poor condition at autopsy because of the high dosage of hormone given, and the histological changes in the brain were more widespread and of a character different from that induced by ACTH. In the cells of the hypothalamic paraventricular and supraoptic nuclei, cortisone caused a reduction in the Nissl material and that which remained was aggregated into clumps which were located in the peripheral part of the cell. In some cells there was moderate vacuolation of the cytoplasm. Nissl material was reduced in the anterior, lateral, ventromedial and posterior hypothalamic nuclei and in the *N. periventricularis posterior pars ventralis*.

Widespread chromatolysis and cytoplasmic vacuolation occurred throughout the thalamus, the following nuclei being affected in all treated rats: *N. anterodorsalis* (Fig. 5 and 6), *N. anteroventralis*, *N. lateralis thalami*, *N. lateralis pars posterior*, *N. parataenialis*, *N. medialis ventralis*, *N. paracentralis*, *N. reuniens*, *N. paraventricularis anterior*, *N. centralis*, and the commissural nuclei. In 2 of the treated rats the following nuclear groups were altered in a similar manner: *N. anteromedialis*, *N. posterior thalami*, *N. parafascicularis*, *N. rhomboidalis*, and *N. paraventricularis posterior*.

No hemorrhage, edema, liquefaction necrosis, or glial proliferation was observed in the cerebral cortex.

Discussion. The observation that ACTH exhibits its greatest effect on the paraventricular nucleus of the hypothalamus correlates well with the results of other studies dealing with this area. Scharrer and Scharrer(13) postulated on the basis of the presence of cytological evidence of secretion by the constituent nerve cells, that they perform both a secretory and nervous function. The failure of dogs with bilateral lesions in the lateral hypothalamic area to show the expected drop in blood eosinophiles during stress combined with other experimental data, suggested to Hume and Wittenstein(14) that the paraventricular nucleus serves as a central area which controls the secretion of ACTH by the anterior hypophysis. Unfortunately, the location of their lesions was not proven by microscopic examination of the brain. Since the supraoptic nucleus gives rise to nerve fibers passing to the hypophysis, it is of interest that destruction of this nucleus in the experiments of Hume and Wittenstein did not interfere with the eosinopenic response and also, that in our experiments ACTH did not modify its structure. These workers concluded that the paraventricular nucleus exerts its control over the hypophysis by humoral rather than nervous means. This conclusion correlates nicely with the cytological changes described here since the reduction in the amount of Nissl material and in size of the nucleolus (structures containing large quantities of ribonucleoprotein) as well as the reduction in cellular and nuclear size might be interpreted in terms of a lowered rate of synthesis of a protein secretion(15). Also, these changes might indicate diminished capacity for discharge of nervous impulses.

Heinbecker and Pfeiffenberger(16) observed degeneration in the paraventricular nucleus in a case of Cushing's syndrome and interpreted this change as being the primary cause of the disease. Our observations on the action of ACTH on the paraventricular nucleus indicate that these workers were dealing with the result of hyperadrenocortical activity rather than with the cause of the disease.

The important rôle played by the hypo-

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thalamus in the regulation of emotional expression is well-known. Hence, these observations may be of some assistance in understanding the changes in personality which sometimes are observed clinically following treatment with these hormones. Since the anatomical modifications induced by cortisone were more general and the rats so treated were in poor condition at the time of autopsy, further studies are being carried on to determine the specificity of the changes elicited by cortisone in the hypothalamus and in the thalamus.

Summary. Administration of ACTH caused chromatolysis in the cells of the paraventricular hypothalamic nucleus. Cortisone affected this nucleus but induced more widespread chromatolysis and vacuolation of thalamic and hypothalamic nerve cells.

Received January 15, 1951. P.S.E.B.M., 1951, v76.

Intraspinal Inoculation of Mice in Experimental Poliomyelitis. (18489)

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A large proportion of the experimental work accomplished in the field of poliomyelitis has been through the use of mice inoculated intracerebrally with the Lansing rodent-adapted virus(1). Low titers, prolonged incubation periods and irregular responses of individual animals have continued to present difficulties in the use of this intracerebral technic(2). The apparent hypersusceptibility of the anterior horn cells of the spinal cord has made it seem obvious that direct inoculation of virus in this location should give better results. Melnick, Horstmann and Ward(3) recognized this and used an intraspinal technic for inoculating monkeys with poliomyelitis materials. However, in spite of a consistently shorter incubation period they concluded that this technic was not practical since a large number of undesirable side reactions occurred and there was evidence that it was a less sensitive method in monkeys than intracerebral inoculation. Howe and Bodian have reported the successful intraspinal inoculation of monkeys with central nervous system virus(4). Other workers in unpublished experiments have attempted intraspinal or intracisternal inoculation of mice through the skin with negative results due to technical difficulties. Using dye solutions the following technic in mice has been developed and its possible application to poliomyelitis research evaluated.

Technic of intraspinal (I.S.) inoculation. Under ether anesthesia the middle of the back of the mouse is wetted down with alcohol and a transverse incision through the skin is made with small iris scissors. The mouse is then held in one hand in such a way that the vertebral column is slightly flexed. Using a ¼ inch 27 gauge short bevelled needle and a 0.25 ml tuberculin syringe the needle point is introduced between the vertebrae at the level of the lumbar enlargement of the cord, slightly to the right of the midline. The direction of the pathway of the needle as slight pressure is applied is at about a 45° angle toward the head and a very slight angle toward the midline. As the point of the needle enters the spinal canal a definite "giving" sensation is felt by the hand holding the syringe. A volume of 0.02 ml is then injected and the needle removed along the same pathway by which it was introduced. No appli-

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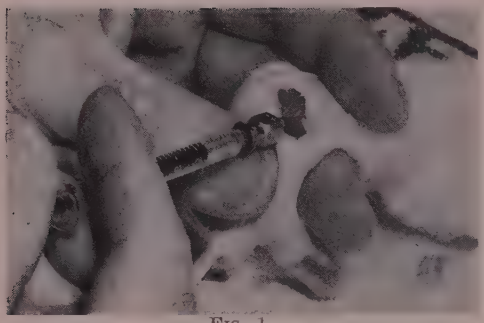

FIG. 1.

cations or dressings are applied to the skin wound which heals without any difficulty. (Fig. 1).

In the preliminary work using dilute aqueous methylene blue as the inoculum, immediately following inoculation dye was observed in the subarachnoid space and the central canal of the cord for varying distances cephalad to the point of inoculation. Once mastered this technic goes rather rapidly. With one assistant anesthetizing the mice, one technician can readily inoculate 100 mice in one hour's time. The age of mice affects the ease with which intraspinal inoculation may be accomplished. Best results seem to be obtained with 4-week-old mice but difficulty of introducing the needle between the vertebrae is not encountered until mice over 6 weeks of age are used.

This is a relatively traumatizing procedure compared with the intracerebral (I.C.) technic. In 2 groups of 200 mice each the mortality during the first 24 hours after intracerebral inoculation was 2% while the same material intraspinally gave 8% traumatic deaths. Occasionally hind leg paralysis will develop as the result of trauma. This almost invariably develops in the first 24 hours and if the animal survives beyond that time the paralysis persists but does not increase unless the animal develops poliomyelitis infection.

Results with Lansing virus. Incubation period and time of death. Lansing infected mouse cords (I.C. passage No. 368) were used to initiate a series of I.S. passages. When relatively heavy inoculum of virus (10^{-1} or 10^{-2} dilutions) were used incubation periods were usually 2 days following intraspinal inoculation. A direct comparison of serial dilutions of virus from the 28th I.S. passage was made I.C. and I.S. using approximately 25 mice per dilution. The results in Table I show the average day of symptoms and day of death at each dilution in the two groups when observed for 35 days after inoculation. The differences in incubation periods are striking. Of a total of 78 mice dying after I.S. inoculation only 6 developed symptoms more than 10 days post-inoculation. In the I.C. group 6 of 92 had incubation periods over 30 days. Deaths in both groups usually occurred within 24 to 48 hours from the onset of paralysis.

Site of paralysis. In Table II a comparison of the location of the paralysis following I.S. and I.C. inoculation of large groups of mice with I.S. Passage No. 28 shows that the proportion of mice dying with preceding paralysis when checked only once daily is about the same in both groups. However, there is a definite reversal in the predilection of site of early paralysis, the I.S. inoculated virus most frequently causing hind leg paralysis while I.C. virus usually involving the front legs first. This difference in the predilection of the site of paralysis by the two methods of inoculation would of course be expected since by I.S. inoculation the virus is deposited in the lumbar section of the cord where innervation for hind leg muscles arises.

Titrations of virus content. A number of comparative titrations of various Lansing virus suspensions (all mouse CNS materials) have been made by I.S. and I.C. technics. In Table III, 14 of these comparisons show that I.S. titers were higher in 13 instances and the average results were 0.7 of a log higher in the I.S. series. All titrations were done with 10 mice per dilution using half log differences in the serial dilutions. In an attempt to evaluate the reproducibility of results of titrations by the I.S. technic the experiment summarized in Table IV was set up. Here serial half log dilutions of I.S. Passage No. 28 Lansing virus were inoculated into 25 mice by I.S. and 25 by I.C. technics but for each dilution were divided into 5 groups of 5 mice each and observed for 35 days separately. This resulted in what was really a titration using 5 mice per dilution but re-

TABLE I. Incubation Period IS Versus IC Inoculation with Lansing Virus.

	Route inoculated	Virus dilutions† 2	2.5	3	3.5	4	4.5	5	5.5	6
Mortality*	IS	—	—	22/24	18/22	12/22	9/25	4/22	7/24	6/22
	IC	24/25	24/24	17/24	10/25	7/25	2/24	6/24	2/27	—
Avg incubation time‡	IS	—	—	2.4	3.8	4.3	5.1	4.7	7.0	18.0
	IC	14.4	15.5	16.7	19.1	17.5	13.0	6.4	8.5	—
Avg death time‡	IS	—	—	4.2	6.0	6.0	7.0	6.0	7.5	18.8
	IC	15.5	17.6	18.9	19.9	18.7	15.5	10.8	9.5	—

* Deaths over total mice inoculated.
† IS mouse passage No. 28 Lansing virus.
‡ Days from inoculation.

TABLE II. Location of Paralysis in IS and IC Inoculated Mice—Lansing Virus.

Route of inoculation*	No. mice	Total dead	Dead with paralysis No.	%	Paralyzed in Front leg first	Hind leg first	Ratio Front leg/hind leg
IS	161	82	46	57	19	33	0.6
IC	200	96	47	49	41	12	3.4

* Inoculated with various dilutions from 10^{-3} to 10^{-6} of IS mouse passage No. 28.

TABLE III. Comparison of Titers of Lansing Virus by IS and IC Technics.

Test	IS	IC
1	5.4	4.5
2	5.0	4.4
3	4.6	3.8
4	4.5	5.0
5	5.1	4.9
6	4.7	4.2
7	4.2	3.7
8	4.0	3.4
9	4.4	3.6
10	4.4	3.3
11	5.1	4.1
12	5.0	4.0
13	4.9	4.2
14	4.4	3.6
Avg	4.7	4.0

peated 5 times. By combining the results of 1, 2, 3, 4 or all 5 of these groups of mice by all possible combinations the results for repeated tests using 5, 10, 15, 20 or 25 mice per dilution could be obtained. In Table IV it is seen that the greatest variation for I.S. inoculation was 0.4 of a log in the 5, 10 and 15 mice per dilution tests and was only 0.1 of a log when 20 mice were used. After I.C. inoculation the greatest variation was 0.5 of a log when only 5 mice per dilution were used but was still 0.2 of a log when 20 mice were employed. As a further check on the consistency of the results by the I.S. technic, two simultaneous titrations by two technicians were performed by both technics. Table V shows the results in two tests to be perhaps slightly more consistent with the I.S. technic.

Distribution of virus between brain and

TABLE V. Comparisons of Titrations by Two Technicians.

Operator	Test No. 1 IS	IC	Test No. 2 IS	IC
A	4.2	3.7	4.4	3.6
B	4.0	3.4	4.4	3.3

TABLE IV. Repeatability of End-points in Titrations of Lansing Virus by IS and IC Technics.

		Combination of groups 1	2	3	4	5
No. mice per dilution		5	10	15	20	25
No. titrations*		5	10	10	5	1
Avg 50% end-points	IS	4.4	4.4	4.4	4.4	4.4
	IC	3.7	3.6	3.6	3.6	3.6
Extreme 50% end-points	IS	4.2-4.6	4.2-4.6	4.2-4.6	4.4-4.5	—
	IC	3.4-3.9	3.4-3.8	3.5-3.8	3.5-3.7	—

* Lansing IS mouse passage No. 28.

TABLE VI. Virus Content of Brain and Cord from I.S. and I.C. Lansing Passage Mice.

		I.S. inoc.				I.C. inoc.			
		Cord titer		Brain titer		Cord titer		Brain titer	
		I.S.	I.C.	I.S.	I.C.	I.S.	I.C.	I.S.	I.C.
I.S.	P30	4.17	4.21	>1.5	>1.5	3.76	3.59	3.28	2.43
I.C.	P379	4.81	4.62	>1.5	>1.5	4.29	4.0	3.15	3.43

cord. Preliminary experiments had indicated that the brains of mice paralyzed after I.S. inoculation of Lansing virus were very low in virus content. In order to determine if this was a characteristic of the I.S. passage virus the experiment summarized in Table VI was performed. Here the 30th I.S. passage virus was inoculated by the I.S. route at a 10^{-3} dilution into one group of mice and I.C. at 10^{-2} dilution in a second group. At 48 hours after inoculation paralyzed mice in each group were killed, the brains and cords being harvested separately. Each pool of brains and cords were then titrated in mice by both the I.S. and I.C. routes. Similarly the 379th I.C. passage Lansing virus was inoculated I.S. at 10^{-2} and I.C. at 10^{-1} and the brains and cords harvested from mice paralyzed at 48 hours, with subsequent I.S. and I.C. titrations. As seen in Table VI with both original passage viruses the brains of mice inoculated I.S. had practically no demonstrable virus whereas the brains of those inoculated I.C. had good titers although not as high as the corresponding cords. There did not appear to be consistent or significant differences in the titers obtained with the I.S. strain and the I.C. strain of virus.

Virus neutralization tests. Since one of the chief applications of a new technic which would shorten the observation time required in the use of Lansing virus in mice would be in routine neutralization tests of serum samples, the I.S. and I.C. technics were compared against two serums. Lansing I.S. Passage No. 28 virus diluted 1/50 was mixed with equal parts of the two serums undiluted and in four-fold dilutions to 1/1024 for I.C. inoculation. The same virus diluted 1/500 was mixed with a second set of similar serum dilutions for I.S. inoculation. One serum specimen was from a monkey hyperimmunized with Lansing monkey cord virus and the other was human serum globulin obtained from a commercial biological laboratory. The two serum samples were inactivated at 56°C for 30 minutes before use. Serum-virus dilutions were held at 4°C for 4 hours then inoculated into groups of 10 mice for each dilution. Virus of each series was also mixed with normal monkey serum (¼ dilution) and after ice-box incubation serial dilutions in this serum were made to determine the titer of the virus used in the test. For the I.C. test 1.4 logs of virus had been used but only 0.9 of a log in the I.S. test. Table VII shows good correlation between the results of neutralization by the two technics.

TABLE VII. Serum Neutralization Tests by IS and IC Inoculation.

	IC	IS
Serum A	1/461†	1/466
Serum B	1/525	1/256
Virus*	$10^{1.4}LD_{50}$	$10^{0.9}LD_{50}$

* Mouse passage No. 28 Lansing virus.
† End-points of serum dilutions giving 50% neutralization of virus.

I.S. inoculation of other laboratory animals. This technic has been relatively easily applied to rabbits, guinea pigs, hamsters and cotton rats and is made less difficult in these species if younger animals are used.

Results with other neurotropic viruses. Titrations by ten-fold dilutions were made by I.S. and I.C. inoculation in parallel series of mice using mouse adapted strains of fixed rabies, eastern and western equine encephalomyelitis, St. Louis encephalitis and choriomeningitis viruses. No differences were noted in titer or incubation period for eastern equine encephalomyelitis or choriomeningitis viruses. Both western equine and St. Louis encephalitis viruses had slightly higher titers and shorter incubation periods by the I.S. route. Rabies virus titered to the same endpoint by both technics but the I.S. inoculation resulted

in a shorter incubation period.

Discussion and summary. A relatively simple technic for the intraspinal inoculation of mice which lends itself to routine laboratory use has been described. For Lansing poliomyelitis virus, it seems to be a more sensitive method of detecting virus than the intracerebral technic. The quantitation of virus titers by this method is consistent between two different technicians and on repeated tests by one technician from any one set of virus dilutions. It can be applied to the routine testing of serums for Lansing antibodies. The chief advantage of the technic is the marked shortening of the incubation period of Lansing infection in mice as compared to that following intracerebral inoculation.

Received January 15, 1951. P.S.E.B.M., 1951, v76.

Effects of Ultrasonic Vibrations on Nerve Tissues.* (18490)

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Numerous studies have already been published concerning the effects of ultrasound on living systems [Gregg(1), Harvey(2,3)]. Among these there are several kinds of experiments on the effects of ultrasound on nerve tissues, both excised(2) and *in situ* (4,5). Further, Lynn and Putnam(6) determined the effect of ultrasound on the brains of mammals, and demonstrated functional disturbances, neurone damage and even death of organisms.

The propagation of ultrasonic vibrations through living tissues is accompanied by a variety of physical factors such as: (1) heating caused by absorption of acoustic energy; (2) periodic pressure changes; (3) radiation pressure; (4) streaming or flow in viscous media; and, (5) high temperatures and pressures associated with cavitation, defined as the formation of holes in liquid media. Any one or all of these factors may produce significant and measurable changes in the state of a living system. The experiments described above do not afford the opportunity to determine how ultrasound produces its effect. The experiments to be described were designed to determine whether tissue heating is a major factor contributing to the effect of ultrasound on nerve tissues.

Materials and methods. The following types of preparations were employed:

(1) The ventral abdominal nerve cord of the crayfish was dissected out in its entirety and immersed in van Harreveld's solution. The sixth abdominal ganglion was always removed and either part or all of the remaining cord was used. The excised nerve cord was mounted in an electrode chamber in contact with 2 glass tubes filled with a salt solution. These salt bridges made contact with small calomel half cells which were, in turn, connected to the amplifier. The spike potentials were amplified by a condenser coupled amplifier and recorded by photographing the trace of a cathode ray tube.

(2) Intact frogs were placed vertically in the path of the sound beam by mounting the animal (dorsal surface down) as firmly as possible on a piece of plywood cut to fit the sound tank. A 2.0 cm hole, centered so

* This research was supported by the Biophysics Branch of the Office of Naval Research under Contract N6ori-71-Navy Task XXI. It is now under the cognizance of the Physiology Branch of the Office of Naval Research.

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FIG. 1.

Spontaneously occurring spike potentials recorded from the commissure between the first and second abdominal ganglia of an excised crayfish ventral nerve cord. Table I indicates the frequency of discharge (average values) as a function of the elapsed time during and after exposure to ultrasound. Records should be read from right to left.

as to fit directly over the crystal, served to admit the sound. The frogs were oriented on the board so that the region of the lumbar enlargement was approximately centered in the aperture. The approximate center of the lumbar enlargement was estimated by adjusting the frog so that a line 12-14 mm behind the posterior edge of the tympanum bisected the hole in the board. The sound tank was filled with distilled water which had been boiled for 10 min. to drive off most of the gas.

(3) Temperature changes in the spinal cords of intact animals, in excised spinal columns containing the cord, and in the ganglia of excised crayfish ventral abdominal nerve cord were measured with constantan-copper thermocouples. For the crayfish ventral nerve cord, a soldered junction made of 0.013 mm copper and 0.038 mm constantan was used. For the spinal cord of the frog, a soldered junction of 0.25 mm copper and constantan was employed. The ventral nerve cord of the crayfish was threaded over the junction and the ultrasound was incident on the preparation in the region of the junction. The thermocouple in the spinal cord was introduced laterally through the foramina of the column through which the peripheral nerves pass. The foramina selected were approximately 12 mm behind the posterior edge of the tympanum. The frogs used in making the temperature measurements were not utilized for any other purpose. The thermocouple output was interrupted by a mechanical chopper, amplified by a condenser coupled amplifier and recorded by photographing the trace of a cathode ray beam. The chopping frequency was 10 per sec. A sudden temperature change, produced by thrusting the junction into a cold water bath, produced a deflection reaching 0.7 of maximum in 0.030 sec. This measurement was made without the chopper. The ultrasound used was at a frequency of about 1 mc and the maximum intensity was 35 watts/cm^2. The vibrations were propagated through water from the generating crystal surface to the preparation. For further details concerning the sound generator, see Fry, *et al.*(7).

Results. 1. *Effect of ultrasound on the ventral nerve cord.* Spontaneous activity recorded from the commissure between 2 adjacent ganglia (1 and 2) of the crayfish ventral abdominal nerve cord, immersed in a balanced

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TABLE I.

Time sequence	Frequency
Control	7.8
Sound on 15″	12
Sound on 17″	7
Sound on 43″	0
Sound off 50″	5.3
Sound off 65″	6.7

salt solution, is illustrated in Record 1, Fig. 1. Superimposed on a low level background activity is a series of periodically occurring spike potentials at an average frequency of 7.8 per sec. Exposure of the 2 ganglia and the commissure to ultrasound (~ 35 watts/cm^2) produces a characteristic sequence of changes in the activity, a typical example of which is illustrated in records 1, 2, and 3 of Fig. 1 and tabulated in Table I. Note that after the sound is turned on, the frequency of the spike potentials at first increases, then decreases and is followed by total disappearance of the large spike potentials after 43 sec. exposure (Record 2). Twenty-five seconds after the ultrasound was turned off, the large spike potentials reappeared, first slowly, then more rapidly, finally reaching a stable frequency of 6.7 per sec (Record 3, Fig. 1). Subsequent treatment of the same preparation with ultrasound produced a similar sequence of events. Similar observations were made on 6 other preparations. Measurements of temperature changes in the ganglion of a preparation similar to the above, under identical conditions, indicated a maximal rise of 1°C.

2. *The effect of ultrasound on the frog spinal cord.* A. Physiological and morphological effects. Twelve intact normal frogs suspended under water at room temperature, 21°-25°C, and placed so that the sound beam was incident on the center of the back over the lumbar enlargement, showed complete paralysis of the hind legs with exposures of 4.3 sec. duration. Shorter exposures to sound either produced no paralysis or a temporary partial paralysis which disappeared after a variable time interval. Experiments similar to the above were performed with frogs cooled and maintained at 1°-2°C. Ultrasound incident on the back over the lumbar enlargement of these cooled frogs produced paralysis of the hind limbs after exposures of 7.3 sec. The paralysis was permanent in all of 50 frogs so treated. Exposures of shorter duration produced no paralysis or a temporary paralysis which disappeared. Stimulation of the sciatic nerves of a frog just after the production of paralysis with ultrasound resulted in muscular response. Similar stimulation of sciatic nerves one week after the paralyzing sound treatment produced no muscular response. Examination of histological preparations of sciatic nerves fixed and stained with osmic acid vapor 2 weeks after irradiation, revealed considerable degeneration of axones. A typical example is illustrated in Fig. 2, No. 2 (compare with control nerve, Fig. 2, No. 1). In all preparations examined, considerable degeneration of axones was evident, as well as degeneration of the region of the spinal cord exposed to the ultrasound.

Histological examination of the spinal cords of ultrasound treated frogs revealed marked abnormality of the large motor neurons of the ventral horn of the gray matter. These abnormalities are evident in spinal cords dissected out and fixed 20 minutes after treatment and stained with thionine ($C_{12}H_9N_3S$), (Fig. 2, No. 4). Note the ragged cell outlines and the very intense stain (compare with control, Fig. 2, No. 3). Normal motor neurons do not stain intensely and have smaller nuclei, usually centrally located. Examination of preparations made four and eight days after ultrasound treatment show neurons which stain intensely and exhibit peripherally located nuclei (Fig. 2, No. 5 and No. 6). These changes are evident in sections both above and below the degenerated regions. A reduction in population of neurones is evident. The eight-day-old lesion, Fig. 2, No. 6, exhibits a marked abnormal appearance. Lower motor neurons from the spinal cords of frogs exhibiting temporary paralysis of the hind legs indicate some abnormality (Fig. 2, No. 7) and those from a frog treated at low temperature for 5 sec. and exhibiting no paralysis of the hind legs (Fig. 2, No. 8) do not show any obvious abnormality.

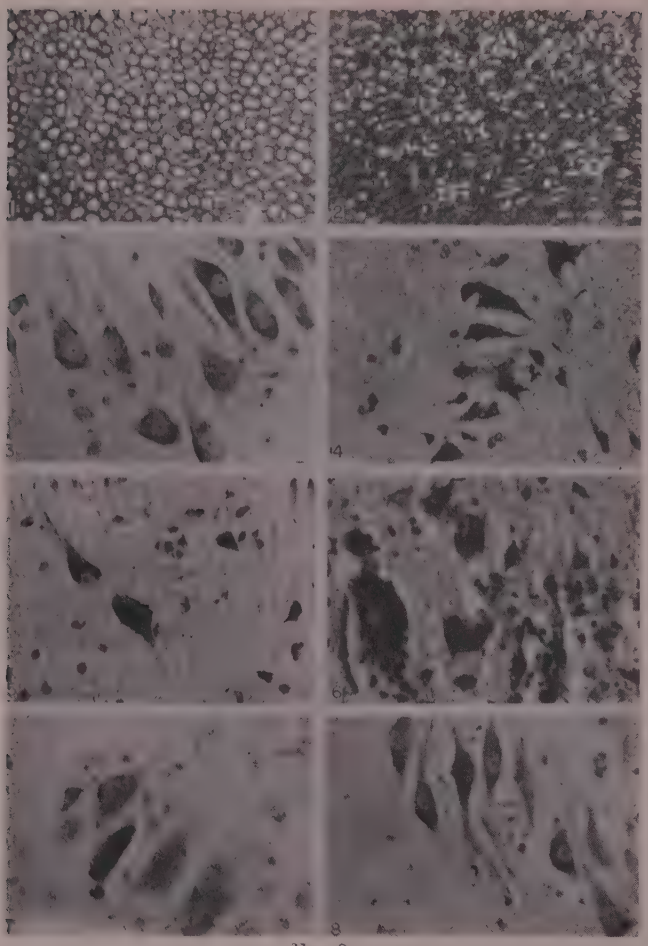

FIG. 2.
Photomicrographs of sections through sciatic nerves (1 and 2) and spinal cords of frogs (3-8) showing neurones in the ventral horns of the gray matter. ×175.

B. Temperature changes produced by ultrasound. Changes in temperature of the spinal cord of intact frogs were measured during and following the period of ultrasound treatment. Ultrasound incident on frogs at 1°-2°C produced a sharp rise in temperature (Fig. 3, Graph 1), which reached a level between 25°-30°C at the end of the 7.3 sec. exposure. After the exposure, the spinal cord exhibits a decrease in temperature. Experiments on isolated spinal column preparations gave similar results. To determine the influence of temperature on the production of paralysis of the hind legs of frogs, 12 experiments were performed using brief repetitive exposure to ultrasound. Frogs, cooled to 1°-2°C, were exposed to ultrasound for 4.3 sec. This is a sub-paralytic dose. The temperature change (max. temp. 15°C) produced by this exposure is indicated in Fig. 3, Graph 2. This exposure was followed by a 4 minute interval to permit the cord temperature to return to the previous level. Then the frog was subjected to a second 4.3 sec. dose of ultrasound, which produced a temperature change similar to the first (Fig. 3, Graph 2). Frogs subjected to a similar procedure (without insertion of the thermocouple) exhibited

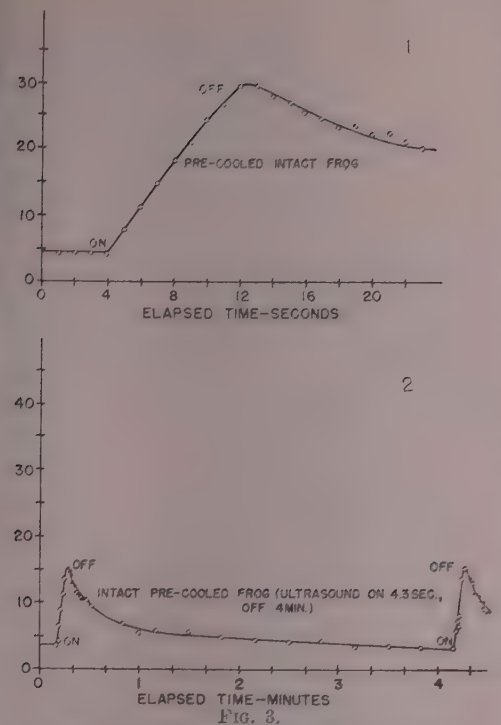

FIG. 3.

Temperature changes as a function of time in the frog spinal cord during and after sound treatment.

permanent paralysis of the hind legs after the second exposure. The effect of temperature on the spinal cord was assessed in the absence of ultrasound. The posterior half of frogs immersed in water baths at 35°C for 20 minutes and then raised to 38°C for 20 minutes showed no obvious abnormality in behavior. Frogs immersed in 40°C water for various periods up to 6 minutes showed a paralysis which gradually disappeared. Temperature measurements in the lumbar region of the spinal cord of frogs in 40°C water indicated a level of 40°C after 6 minutes.

Discussion and conclusions. Whenever ultrasound is transmitted through living tissues the existence of damaging temperature levels, produced by absorption of acoustic energy, must be ascertained. Ultrasound incident on ganglia of the crayfish ventral nerve cord containing spontaneously active neurones caused a reversible depression of the spontaneous activity. A maximal and rapid temperature increase of 1°C was measured. Prosser(8) has shown that increasing the temperature 1°C between 26°-30°C may produce an increase in the frequency of discharge of single units of about 4-5 per sec. The effect of ultrasound in depressing the frequency of discharge is in a direction opposite to the effect of the temperature change. It is concluded, therefore, that the effect of ultrasound on these spontaneously active neurones is mediated by physical factors other than the simultaneously occurring but slight temperature change.

Examination of the data pertaining to neurone damage in the frog indicates that paralysis can occur in the absence of high (35°-40°C) temperature levels. For instance, frogs pre-cooled to 1°-2°C and subjected to a 7.3 sec. dose of ultrasound never exhibit temperature levels in excess of 30°C (Fig. 3). Further, exposure of pre-cooled frogs to successive sub-paralytic doses (4.3 sec. exposure, followed by a four minute interval) show temperature maxima of 15°C (Fig. 3), and yet, paralysis occurred after two exposures. The initial rates of change of temperature measured in the intact frog spinal cord, are rather constant, 1.8°C/sec. It is possible that the rapid increase in temperature occurring in the spinal cord upon incidence of ultrasound may produce physiological changes even though the final level of temperature remains below 30°C. Since rates of temperature change of the order of 1.8°C/sec. could not be achieved except by ultrasound, it was not possible to assess this factor. Unless a rate of change of temperature of the order of 1.8°C/sec. exerts a specific effect leading to paralysis it is apparent that the temperature changes measured cannot account for the experimental results in all cases. It is concluded that ultrasound produces effects on the neurones of the spinal cord which are primarily dependent upon physical factors other than temperature, such as periodic pressure changes, particle acceleration, radiation pressure and temperature and pressure changes associated with cavitation.† The evaluation of some of these factors must be

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left to future research.

Summary. The excised crayfish ventral nerve cord exposed to ultrasound (~35 watts/cm², frequency 1 mc) exhibited a reduction of spontaneous activity after several seconds exposure and recovered its original activity about one minute after the ultrasound was turned off. Frogs positioned so that ultrasound was incident on the dorsal surface over the lumbar enlargement of the spinal cord exhibited paralysis of the hind legs after 4.3 sec. exposure (at room temperature) and exhibited paralysis after 7.3 sec. exposure (at 1°-2°C). Histological examination of the sciatic nerves showed extensive degeneration of nerves and examination of the spinal cord indicated destruction of the lower motor neurones. It was concluded that the effect of ultrasound on the systems studied is produced by physical factors other than temperature.

We would like to extend our appreciation to Professor C. L. Prosser for his continual interest and helpful suggestions during the course of this research. We also wish to thank Professor Warren McCulloch for his interest in this work.

Received December 19, 1950. P.S.E.B.M., 1951, v76.

[*] Cavitation is a name applied to the phenomenon of formation of holes in liquid media.

Effect of Citrovorum Factor in Pernicious Anemia.* (18491)

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(Introduced by Wm. Dock).

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Although Addisonian pernicious anemia is therapeutically well controlled by the parenteral administration of vit. B_{12}, the exact mechanism of response, and particularly the relationship of vit. B_{12} to pteroylglutamic acid (PGA), is still not well defined. Because of bacterial interrelationships of the citrovorum factor (CF) to vit. B_{12}, PGA, and thymidine, and because of the hematopoietic activity of vit. B_{12}, PGA, and thymine in pernicious anemia, it was decided to study the clinical and hematologic effect of CF in patients with pernicious anemia. CF(1) is a substance first obtained from liver extracts active against pernicious anemia, the unit of which has been defined as that amount of substance required for half maximal growth of *Leuconostoc citrovorum* 8081 in 72 hours on a basal medium. *L. citrovorum* shows some growth response to thymidine(2-5) or to large amounts of PGA, but none to vit. B_{12}(4,5). CF can replace PGA as a growth factor in all organisms that have been tested. Acid hydrolysis destroys the citrovorum activity, leaving the PGA activity unchanged(6,7). CF can be obtained from liver and yeast extracts(1,5) and has been isolated from the urine of rats and humans given PGA(8,9). Its biological synthesis by rat liver slices is

* Presented at annual meeting of American Federation for Clinical Research, Eastern Section, Washington, D.C., Dec. 9, 1950.

The expenses of this investigation were defrayed by a Lederle grant.

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TABLE I. Citrovorum Factor: Hematological Response in Patients with Addisonian Pernicious Anemia.

Case No.	1	2	3	4	5	6
Citrovorum factor, mill. units I.M. daily	5	5	5	10	10	10
Initial RBC, mill./cu mm	1.46	2.55	2.19	1.30	2.65	1.75
RBC, mill./cu mm at 3 wk	3.09	3.56	3.27	2.51	3.75	
Expected RBC at 3 wk, mill./cu mm	3.15	3.69	3.54	3.04	3.76	
Day of reticulocyte peak	10	10	11	8	7	None
Max. reticulocytes, %	12.7	5.4	6.6	6.9	9.8	
Expected max. reticulocytes, %	24.5	10.0	16.0	27.0	9.0	20.5

* Subsequently received 120 γ B_{12} I.M. and responded with reticulocyte and RBC rise.

augmented by PGA and ascorbic acid(10). Folinic acid, a substance with CF activity, has been extracted from hog liver and synthesized chemically by hydrogenation of formylfolic acid(11-13). In experiments studying the inhibitory growth effect of the PGA antagonists, aminopterin and amethopterin, on *L. citrovorum*(7,14,15), *Strep. faecalis*(7), mice(7,16,17), chick embryos(18), and mouse leukemia(19), it was found that CF was very active in antagonizing the inhibition in a competitive manner. This was in contrast to PGA which gave little or no protection except when administered in large amounts, and to vit. B_{12} which showed no effect whatsoever. Thymidine, however, reversed aminopterin toxicity, but did so in a non-competitive fashion.

In an attempt to learn more about CF relationships, we undertook a study of the effect of CF in pernicious anemia patients, both with and without the addition of aminopterin.

Material and methods. Ten patients, ages 45 to 80 years, with Addisonian pernicious anemia hospitalized on the medical services of Kings County Hospital were studied. All received the usual hospital diet. In all cases the marrows were megaloblastic and histamine-fast achlorhydria was present. Red blood cell, white blood cell, hemoglobin, and hematocrit determinations were done 3 times a week on venous blood with Wintrobe oxalate mixture. Reticulocyte values were determined daily. The CF used† contained 20 million units per ml and was diluted with physiologic saline for doses of less than 10 million units. It was administered intramuscularly in all cases.

Results. The data on those patients who received CF alone are recorded in Table I. Where transfusions were given before therapy, the initial red blood cell counts noted are those after transfusion. Of the first 5 cases, only case 5 (Fig. 1) had a maximal reticulocyte response. This patient received 10 million units daily. Case 4, on the same dosage, had a submaximal reticulocyte peak and a submaximal rise of red blood cells at the end of 3 weeks. However, all 3 cases (Case No. 1, 2, 3) who received 5 million units daily had maximal red cell rises at 3 weeks despite submaximal reticulocyte peaks. All 5 patients had conversion of the marrow to a normal normoblastic one while on therapy.

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† The CF ("Leucovorin") was kindly supplied to us by Dr. J. M. Ruegsegger and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. Twenty million units per ml is equivalent to 3 mg CF per ml.

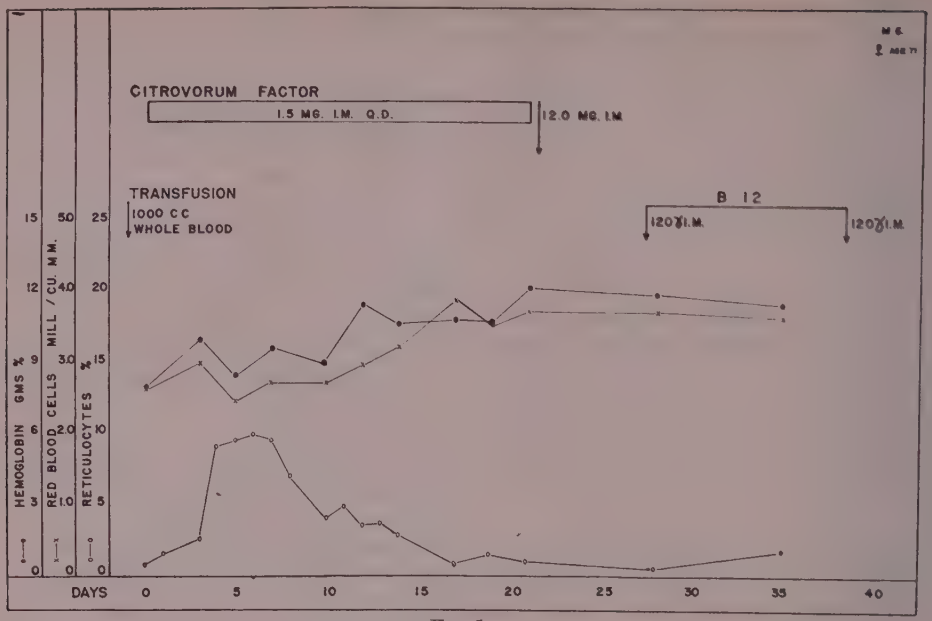

FIG. 1.

All had satisfactory clinical improvement, although it seemed somewhat less dramatic than that expected with vit. B_{12} or liver extract therapy. The glossitis in Case 3 was unaltered at the end of 3 weeks. There was no change in the neurological status in any of the patients during the 3 weeks of therapy.

However, one patient (Case 6) showed no response to CF in a dosage of 10 million units daily. On the eighth day of treatment she had a reticulocyte count of 0.1% and a falling red blood cell count, hemoglobin, and hematocrit, with a megaloblastic marrow. She subsequently responded to single intramuscular injection of 120 γ of vit. B_{12}.

In order to study maturation effect *in situ* in the bone marrow, one patient (Casel) received 10 million units of CF intrasternally. At the end of 48 hours there was partial conversion of the megaloblastic marrow to a normoblastic one at the same site, but a specimen of marrow aspirated simultaneously from the iliac crest showed a similar state of maturation.

Four additional patients were given aminopterin in addition to specific therapy with PGA and vit. B_{12} in an attempt to inhibit their hematopoietic response, so that subsequent administration of CF could be studied for its possible antagonistic effect. Case 7 (Fig. 2) received 0.125 mg aminopterin and 7 mg PGA intramuscularly on alternate days for a 10 day period. (This amount of PGA when given alone is expected to produce optimal hematopoietic response in a pernicious anemia patient). In this case there was a submaximal rise of 10.9%. Furthermore, there was a secondary reticulocyte peak of 6.1% in the second 10 day period during which time 20 million units of CF every other day was substituted for the PGA. Case 8 (Fig. 3) received twice the amount of aminopterin (0.25 mg) and the same amount of PGA (7 mg) every other day for a 10 day period with no reticulocyte response. Subsequent administration of CF in place of the PGA resulted in a submaximal reticulocyte peak of 6.9% on the 9th day.

Two additional patients were given vit. B_{12} simultaneously with aminopterin. One patient received 0.25 mg aminopterin, 30 γ vit. B_{12}, and 15 mg PGA intramuscularly every other day for a 10 day period. The other patient received 1 mg aminopterin and 10 γ vit. B_{12} on alternate days for a period of 10 days. Since both patients had a maximal reticulocyte response, CF was not given in a later period.

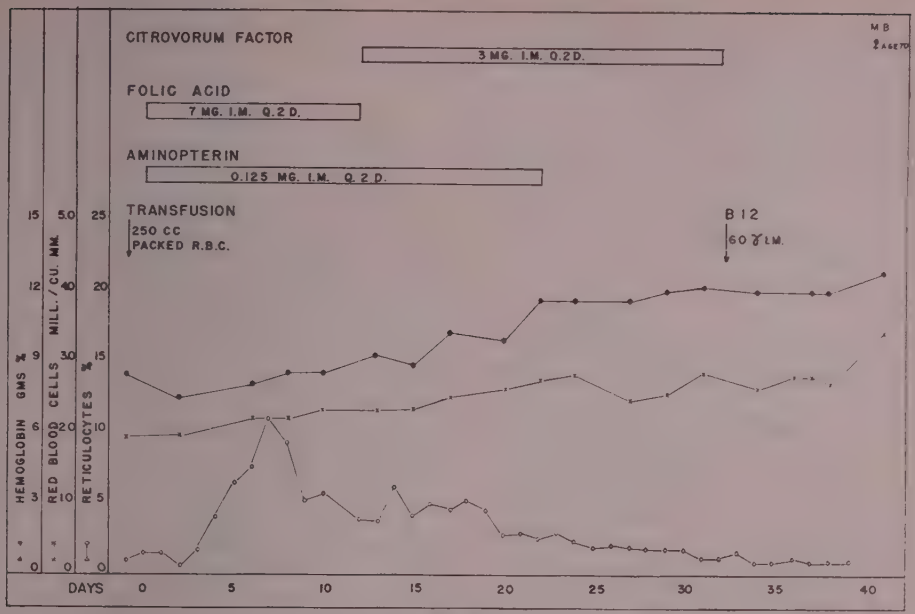

FIG. 2.

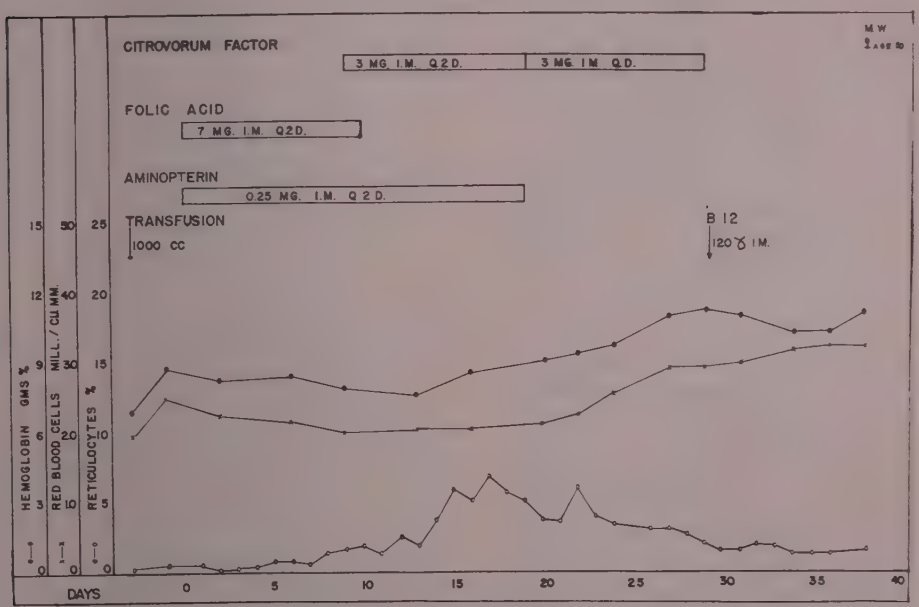

FIG. 3.

Discussion. Five of 6 patients with pernicious anemia gave satisfactory responses to the daily intramuscular administration of 5 to 10 million units (0.75 to 1.5 mg) of CF. In view of the fact that 4 cases gave maximal red cell rises at the end of 3 weeks, this amount might be considered to be equivalent to 1 U.S.P. unit of liver extract. However, the reticulocyte responses were only about half that expected, with the exception of one maximal response. Although a lack of correspondence between reticulocyte and red blood cell

response in pernicious anemia patients treated with liver extract has been reported by Clark (20), the reticulocyte responses seen here seem unusually poorly correlated with the erythrocyte responses, so that it may be possible that CF is relatively lacking in specific reticulogenic activity. Studies are in progress to determine whether daily doses of less than 5 million units of CF can produce a maximal erythrogenic response. The fact that one of the patients failed to give any response to 10 million units daily of CF is of considerable interest, but cannot be explained at the present time. Therapeutically and hematologically CF is not as dramatically effective as vit. B_{12} in the treatment of Addisonian pernicious anemia. This might be expected because of the closer chemical similarity of CF to PGA. For this reason, it will be of interest to observe the effects of CF in megaloblastic anemias associated with the presence of free HCl, particularly in the rare cases refractory to both PGA and to vit. B_{12}. CF has been ineffective in one patient with macrocytic anemia due to liver disease and in one patient with refractory macrocytic anemia, in both of whom the marrow was normoblastic. The lack of local maturation effect on megaloblasts in the marrow after intrasternal instillation of CF in one of our cases corresponds to the negative effect of PGA(21) under similar circumstances, and is in contrast to the local maturing action of vit. B_{12}.

Of considerable interest has been the effectiveness of CF in 2 of our patients in reversing the inhibitory effect of aminopterin. In one case PGA was without any effect, and in another case PGA was only partially effective, with amounts given on a comparable weight basis to the CF. However, we have not yet used amounts small enough to check the ratio determined by Burchenal *et al.* (19). These investigators found 625,000 to 1,250,000 units (0.18 to 0.33 mg) per kg of CF to be approximately as active as 15 mg per kg of PGA in preventing the antileukemic effects of amethopterin in mouse leukemia. This is a ratio of about 1:50 of CF to PGA on a weight basis.

Aminopterin had no inhibitory effect on the response of the two patients who received vit. B_{12}. This is in contrast to the finding of Meyer and associates(22), and may be due to a relatively insufficient amount of aminopterin. However, there is no reason to assume that the aminopterin-induced physiological changes are directly comparable to the defects present in pernicious anemia. Furthermore, it is quite possible that PGA and vit. B_{12}, although arriving at the same hematological endpoint in pernicious anemia, do so through distinct though related routes.

Summary. 1. Citrovorum factor, in amounts of 5 to 10 million units daily intramuscularly, has been shown to produce adequate hematologic and clinical response in 4 of 6 patients with Addisonian pernicious anemia, and a submaximal response in another. A 6th patient failed to respond altogether. 2. Local marrow instillation of citrovorum factor failed to produce enhanced maturation of megaloblasts *in situ*. 3. Citrovorum factor has been found to be more effective than pteroylglutamic acid in reversing aminopterin inhibition in patients with pernicious anemia.

We wish to express our gratitude to the members of the medical house staff, whose cooperation made this study possible.

Addendum. Although Bethell and coworkers (J. Lab. Clin. Med., 1948, v 33, 1477.) found that the simultaneous administration of 1 mg aminopterin and 1 γ vit. B_{12} daily for 16 days did not cause a hematologic response, this result may be due to the relatively small amount of vit. B_{12} administered.

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Received January 22, 1951. P.S.E.B.M., 1951, v76.

Thyroactive Iodinated Protein in Protein-Depletion of Rats for Use in Protein Assays. (18492)

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Since the development by Cannon and his associates(1,2), of the use of the hypoproteinemic rat in studies on the dietary utilization of proteins and of amino acids, the general technic has been employed in many laboratories for assaying the plasma protein-regenerating ability of proteins and of amino acid mixtures. However, the method has the disadvantage that a relatively long time, usually about 3 months, is required for the preparation of the animals for purposes of assay. In view of the accelerating effects of thyroid hormone on protein metabolism, which have been well described by various investigators(3), the feeding of one of the thyroactive substances, such as an iodinated protein, would appear to be a useful means of speeding up the depletion process in the preparation of test rats. Data presented here were obtained from some preliminary and exploratory trials made to test this possibility and indicate that the use of iodinated casein for this purpose is practical.

Observations were made on the changes in plasma proteins in rats fed for different periods of time on a basal low-protein diet and in rats fed the same basal diet supplemented with 0.1% and 0.15% of iodinated casein.* Further observations were made after a period of repletion, on one of the groups of animals which had been depleted with iodinated casein. Male albino rats of approximately 200 g weight were used in all groups. The animals were housed individually and fed the diets *ad libitum*. The diets used are indicated in Table I. The iodinated casein added to diets 2 and 3 was mixed directly with the other ingredients. Blood samples of 0.3 to 0.4 ml amounts were obtained from the rats by heart puncture and mixed with a small amount of purified heparin. The blood was centrifuged and total protein determined on the clear plasma, using the falling drop technic of Barbour and Hamilton(4).

TABLE I. Composition of Diets Used.

Ingredients	Diets 1	2	3	4
	%	%	%	%
Equal parts sucrose and dextrinized starch	83.8	83.7	83.65	61.8
Agar	5	5	5	5
Lard	4	4	4	4
Casein	—	—	—	22
Salt mixture	4	4	4	4
Liver conc.	0.2	0.2	0.2	0.2
Irradiated yeast	3	3	3	3
Iodinated casein	—	0.1	0.15	—

Additional vitamins were included, per k, in all diets as follows: Thiamine 1.08 mg, riboflavin 1.63 mg, niacin 2.64 mg, pyridoxine 1.20 mg, calcium pantothenate 2.55 mg, 90% beta carotene mixture 20 mg, alpha tocopherol 24 mg.

The results are summarized in Table II. These data show the effect of iodinated casein in reducing the time necessary for depletion. Gross appearance and weight changes of the animals corresponded to the changes in plasma protein shown by the various groups in the table.

Rats in Group 5, after having plasma proteins depleted by 3 weeks feeding on diet 2 to a level 1.3 g below that of the controls, were then repleted by 7 days of feeding 1.8 g per day of crude casein in addition to the basal low protein diet. These animals gained about 4 g per day in weight and the regenera-

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* Iodinated casein (Protamone) labeled to contain activity equal to 3% thyroxine obtained from the Cerophyl Laboratories, Kansas City, Mo.

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TABLE II. Effect of Thyroprotein on Plasma Protein.

Animals		Diet		Time on treatment, weeks	Plasma protein	
Group No.	Total No.	No.	Thyroprotein level, %		Mean, %	S.E. of mean
1	6	4	0	16	7.26	.25
2	5	1	0	4	6.08	.02
3	5	1	0	10	5.15	.15
4	4	2	.1	2	5.89	.06
5	4	2	.1	3	5.92	.14
6	4	2	.1	4	5.32	.16
7	3	2	.1	6	4.94	.38
8	4	3	.15	4	4.46	.21
9*	4	1†	0	1	7.12	.46

* These were depleted animals from Group No. 5.

† For a repletion period of 1 wk they were fed, in addition to Diet 1, enough casein to supply .24 g N/day.

tion of plasma protein occurred equally as rapidly as in other experiments in which rats were similarly repleted after bring depleted without thyroactive protein.

This modification of the Cannon technic has been applied to mixed feeds containing both animal and vegetable proteins. In this assay trial, nitrogen intake was kept constant. Commercial casein and blood fibrin, as standards representing good quality protein, raised the plasma protein 1.10 g and 1.29 g respectively. A mixed feed which had given questionable results with other animal species raised the plasma protein only .59 g. So far as the method was concerned the only effect of iodinated casein appeared to be to speed up reduction of body weight and plasma protein.

Received December 5, 1950. P.S.E.B.M., 1951, v76.

Comparison of Cardiac Action of Bufalin, Cinobufotalin, and Telocinobufagin with Cinobufagin. (18493)

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Ch'an Su is a commercial preparation of the Chinese toad venom employed in medicine for centuries. Previous investigations in this laboratory(1-3) resulted in the isolation of two digitalis-like principles, cinobufagin and cinobufotoxin. The source of Ch'an Su was proved to be the parotoid secretion of *Bufo bufo gargarizans*(4). The chemical structure of cinobufagin was elucidated by Tschesche and Offe(6) and Jensen(6). From a supply of Ch'an Su from this laboratory Meyer(7) in T. Reichstein's laboratory, succeeded in isolating 6 well-characterized substances, all having a digitalis-like action. They are cinobufagin, bufalin, bufotalin, cinobufotalin, gamabufotalin (gamabufagin), and telocinobufagin. Bufalin, cinobufotalin, and gamabufotalin were previously isolated by Kotake and Kuwada(8). Bufotalin is identical with

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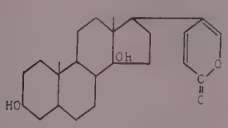
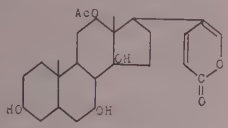
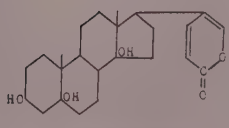
the principle which occurs in the venom of the common European toad, *Bufo vulgaris*. Telocinobufagin is a new substance. Through the cooperation of Prof. T. Reichstein of the Pharmaceutical Institute of the University of Basel, we had access to cinobufagin, bufalin, cinobufotalin, and telocinobufagin for a pharmacological study. The structural relationship of the four substances, based on the work of Meyer(7) and Kuwada and Kotake (9), is as follows:



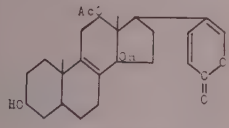
Bufalin



Cinobufotalin



Telocinobufagin



Cinobufagin

The formulas of cinobufotalin and cinobufagin are tentative. It must be stated that the cinobufagin originally reported on from this laboratory(1-3) was a molecular compound of constant composition and melting point. It contained 2 parts of cinobufotalin and 1 part of a second substance. Kotake and Kuwada (8) retained the term cinobufagin for the latter. It was only by chromatography that the two could be separated(7). The sample of cinobufagin in this investigation was therefore a single substance in contrast with the molecular compound which we previously studied.

Our principal interest was to compare their activity on the heart. All 4 substances required ethanol for solution. The stock solution of each was 0.1% with 47.5% of ethanol. Dilutions of this stock solution in water or saline were clear—suitable for animal experiments.

When a dose of 0.25 to 0.5 mg (total) was

TABLE I. Potency in Cats.

Cinobufagin			Telocinobufagin			Cinobufotalin			Bufalin		
Sex	Wt, kg	Dose to kill, μg per kg	Sex	Wt, kg	Dose to kill, μg per kg	Sex	Wt, kg	Dose to kill, μg per kg	Sex	Wt, kg	Dose to kill, μg per kg
M	2.513	264.6	F	2.336	102.7	M	2.374	155.0	M	2.060	183.5
F	2.819	119.9	F	2.460	100.0	F	2.798	120.4	M	2.227	148.6
M	2.710	260.9	M	1.668	99.5	F	2.750	133.1	F	2.062	177.5
F	2.043	243.3	F	2.139	102.4	M	2.542	329.7	F	1.938	96.0
M	2.448	257.4	M	2.482	110.0	F	1.864	279.0	F	2.293	145.7
F	2.192	158.8	M	2.887	122.6	M	2.628	188.7	F	2.054	158.7
M	1.624	142.2	M	2.646	70.7	M	2.112	276.5	F	2.585	91.3
F	1.612	212.8	F	2.114	151.4	F	2.148	185.3	F	2.335	118.6
M	1.646	243.0	M	2.242	82.1	M	2.480	135.1	F	2.414	137.1
M	1.868	183.1	M	2.639	94.7	F	2.076	326.6	M	2.448	145.4
Mean (geo.)					101.6 ± 6.6			199.0 ± 24.4			137.0 ± 10.2
L.D. ± S.E. 201.6 ± 18.1											

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injected into the lymph sac of the common frog, *Rana pipiens*, weighing between 25 and 30 g, systolic standstill resulted as observed in an hour. In etherized cats a 1:100,000 solution injected intravenously at the rate of 1 cc per minute produced slowing of heart rate, followed by irregular pulse, secondary tachycardia, and finally ventricular fibrillation which was the cause of death. Electrocardiograms recorded from the barbitalized dog during the injection of a 1:50,000 solution at the rate of 1 cc per minute revealed changes typical of digitalis administration. Bradycardia, P-R prolongation, inversion of T-wave, ectopic rhythm, ventricular tachycardia, bundle branch block, and ventricular fibrillation, occurred in order. The results were similar with all 4 substances.

Each substance was then assayed in etherized cats by the intravenous infusion of a 1:100,000 dilution at the rate of 1 cc per minute until death occurred—10 cats for each substance. The mean (geometric) lethal dose of the present sample of cinobufagin in 10 cats was found to be 0.2016 $\pm$ standard error of 0.0181 mg per kg. When compared with the mean (geometric) lethal dose of the sample of cinobufagin previously isolated in this laboratory(10), which was 0.2191 $\pm$ 0.0115 mg per kg in 44 cats, the difference was statistically not significant. In other words, cinobufagin as a single substance has approximately the same potency as its molecular compound of cinobufotalin and itself.

Protocols are summarized in Table I. The potency of cinobufotalin, as judged by the mean lethal dose, is very close to that of cinobufagin. Both bufalin and telocinobufagin are decidedly more potent than cinobufagin. Telocinobufagin is the most potent of all four substances studied in this investigation.

It is obvious that the presence of the acetoxy group at C_{12} diminishes the cardiac activity since both cinobufotalin and cinobufagin are weaker than bufalin and telocinobufagin (see formulas above). The occurrence of an OH group at C_5 definitely confers a higher potency on telocinobufagin as compared with bufalin. No significant difference in activity occurs when a double bond appears between C_8 and C_9 in the molecule of cinobufagin, and an OH group replaces the H-atom at C_7 of cinobufotalin. All four substances have a higher activity on the cat's heart than many digitalis-like glycosides(10). This may be attributed to the 6-membered lactone ring in their structure, just as hellebrigenin exceeds its glycosides in activity(11). Conjugation with sugar molecules at the OH group of C_3 will very likely render bufalin, telocinobufagin, cinobufagin, or cinobufotalin less potent.

Like cinobufagin which was previously reported(3), bufalin, cinobufotalin, and telocinobufagin injected intravenously, induced vomiting in non-anesthetized cats, in the dose of one-half of the median lethal dose as determined in etherized animals. Sublethal doses of either substance rapidly injected by vein raised the carotid blood pressure. Contraction of isolated rabbit intestine immersed in Tyrode's solution took place when a small amount of either of the three substances was added to the bath.

Summary. The cardiac activity of bufalin, cinobufotalin, telocinobufagin and cinobufagin has been compared. They all have a digitalis-like action. In etherized cats the order of activity from high to low is telecinobufagin $>$ bufalin $>$ cinobufotalin $=$ cinobufagin.

The authors are indebted to Misses Nila Maze, Marian H. Ellaby, Eva Sommermeyer, and Messrs. Harold M. Worth, John S. Welles, and Harold E. Roeder for their invaluable assistance in these experiments.

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Received January 22, 1951. P.S.E.B.M., 1951, v76.

Failure of Histamine-Beeswax Mixture to Produce Gastrointestinal Lesions in Dogs after Total Gastrectomy.* (18494)

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It has been demonstrated by numerous investigators that ulcers of the stomach, duodenum, and, to a much lesser extent, the remaining portions of the gastro-intestinal tract, occur consistently after the daily or frequent injections of a histamine-beeswax mixture in dogs and other laboratory animals(1). This method has been utilized for the evaluation of various pharmacologic preparations and for the study of surgical alterations of the stomach and duodenum in relation to the etiology and pathogenesis of peptic ulcer. Thus, it has been shown that a three-quarter gastrectomy with a short duodenal loop will prevent the occurrence of histamine-induced ulcers(2). Ulcer formation after histamine-beeswax injections was delayed or prevented by administering sodium lauryl sulfate, a pepsin inhibiting substance, twice daily(3), or by a Sippy dietary regimen(4). That a disturbed motility is not concerned in this type of experimental ulcer production is indicated by reports that neither atropine(5) nor bilateral vagotomy(6) prevent histamine-beeswax ulceration. It has been shown that postganglionic sympathectomy of the upper gastrointestinal tract through excision of the celiac, or of the celiac and superior mesenteric ganglia, accelerates the production of these histamine provoked lesions(7), and that preganglionic sympathectomy does not alter the sensitivity of dogs to histamine ulcerations(8). Venous stasis induced by ligation of the splenic and portal veins increases this type of ulcer formation(9), as does chronic arterial spasm induced by epinephrine injections(10), hemoconcentration after severe burns(11), and experimental fatty embolism of the gastro-duodenal arterial system(12).

Most observers believe that peptic ulcer formation is due to excessive peptic acid activity, although the factors of local tissue susceptibility cannot be ignored(13). Recently we have observed(14) varying degrees of ulceration in the intestinal tract of dogs (jejunum, ileum, and colon) following the daily injection of a histamine-beeswax mixture. Perhaps these ulcers should be considered the result of an increase in volume of gastric secretion caused by histamine stimulation with insufficient neutralization (buffering)(15) and subsequent stasis in the small intestine and colon, thus allowing its proteolytic action to continue unchecked. Pos-

* Supported by U.S.P.H. Grant RG-321.

† Clinical Fellow, American Cancer Society.

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TABLE I. Necropsy Results After Daily Intramuscular Injections of Histamine-Beeswax in Dogs Following Total Gastrectomy.

Dog No.	Date of oper.	Wt, kg	Date inj. begun	Wt, kg	No. of inj.	Wt at necropsy	Pathological changes				
							Esoph.	Duo.	Jej.	Ileum	Colon
37	12/6	9.8	12/29	7.9	34	7.4	0	0	0	+	0
42	10/31	7.8	12/16	5.9	21	4.5	0	0	+	+	0
46	12/6	7.1	12/29	5.7	34	5.1	0	0	0	0	0
53	1/6	10.7	1/20	9.4	31	5.5	0	0	0	0	0
54	4/19	11.1	5/20	8.3	40	—	0	0	0	0	0
64	1/9	10.2	1/23	8.8	40	9.2	0	+	0	0	0
84	5/25	13.7	6/8	9.9	13	9.7	0	0	0	0	0
110	8/19	11.4	9/18	9.5	40	7.4	0	0	0	0	0

Pathological changes: 0, no change noted; +, slight hyperemia only.

sibly significant are the effects of histamine-beeswax on pancreatic and small intestinal secretions, and its angiotoxic action(16).

Our attempt to clarify the role of gastric juice in the pathogenesis of histamine-beeswax induced gastrointestinal ulcers concerns observations made on 8 healthy mongrel dogs subjected to transthoracic total gastrectomy. Several weeks after operation, depending upon their recovery and their ability to eat and retain food, the dogs were given daily intramuscular injections of histamine-beeswax (2.5 mg of the histamine base per kilogram of animal weight), prepared according to the original description of Code and Varco(17). Injections were administered daily for periods varying from 21 to 40 days. The histamine-beeswax mixture was first tested for its efficacy on total gastric pouch dogs. The animals were maintained on a semi-soft diet of milk, protein supplements, eggnog, and ground meat. They were fed several times during the day. The histamine mixture was injected late in the day and following the injection all food was removed from the animals' cages. The animals were sacrificed at varying intervals after receiving the histamine-beeswax mixture. Complete necropsy was performed with particular scrutiny of the gastro-intestinal tract. The gross observations were supplemented by microscopic examinations. The results are summarized in Table I.

In no instance was any evidence of erosion or ulceration of the esophagus, small intestine, or large intestine present. This supports the contention that ulcerating lesions observed in the small intestine and large intestine of intact dogs after repeated injections of histamine-beeswax are dependent upon the action of gastric juice, and that other possible modes of histamine action are not prominent, if present at all.

Conclusions. 1. Eight dogs were subjected to total transthoracic gastrectomy and following this were given daily intramuscular injections of a histamine-beeswax mixture for periods varying from 21 to 40 days. 2. In no instance did any ulcers of the esophagus, small intestine or large intestine develop. 3. This supports the hypothesis that histamine-induced ulcers of the gastro-intestinal tract of the intact dog are due to the excessive production of gastric juice.

Received January 17, 1951. P.S.E.B.M., 1951, v76.

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Disintegration of Excised Fish Gills in Low Concentrations of Sodium Chloride. (18495)

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During the course of experiments on the oxygen consumption of excised gills of goldfish, it was observed that gills respiring in 1 mM sodium chloride apparently had a lower Q_{O_2} and seemed to disintegrate to a greater degree than gills in 125 mM sodium chloride. Consequently, the following experiments were conducted in an attempt to determine some of the mechanisms responsible for the difference.

Experimental methods. The goldfish (weighing 20-30 g) were pithed, the right and left second gill arches excised, washed free of superficial blood and transferred to chilled Warburg flasks containing 2 ml of an appropriate oxygenated medium carrying 100 mg of glucose per 100 ml of solution. Clots in the afferent and efferent vessels were effective seals that retained the blood trapped in the gill capillaries. The left gill served as a control for the right gill from the same fish. The Warburg flasks containing the gills were flushed with oxygen, placed in a water bath (37°C), and 20 minutes allowed for equilibration. Readings were made at the end of 30 and 60 minutes. Oxygen consumption was calculated on the basis of the dry weight of the filaments. Some of the gill arches were subjected to the same routine as in the metabolism studies, but at the end of one hour, were fixed in Lavdowsky fluid, sectioned and stained with muscicarmine-haematoxylin or hematoxylin-eosin.*

Results. Experiments with sodium chloride. Gills respiring in 1 mM sodium chloride solution at 37°C consumed 5.6 ± 0.46 mm^3 of O_2/mg dry wt/hr (mean of 24 fish) as compared to 8.9 ± 0.49 mm^3 of O_2/mg dry wt/hr (mean of 24 fish) for the gills in 125 mM sodium chloride (Fig. 1). Both gross and microscopic examination revealed an obvious breaking apart of the gill filaments in the 1 mM solution. Intact cells were observed separated from the filaments and in addition, many free nuclei were present. Neither of the latter observations was noted in the tissue in the 125 mM solution. The difference of the two means was highly significant, $t = 10.54$ and $P < 0.001$ (difference of correlated means as given by McNemar)(1). While oxygen utilization was nearly a linear function of time in the stronger solution, there was a marked decline in the rate of oxygen consumed during the last 30 minutes in the more dilute medium.

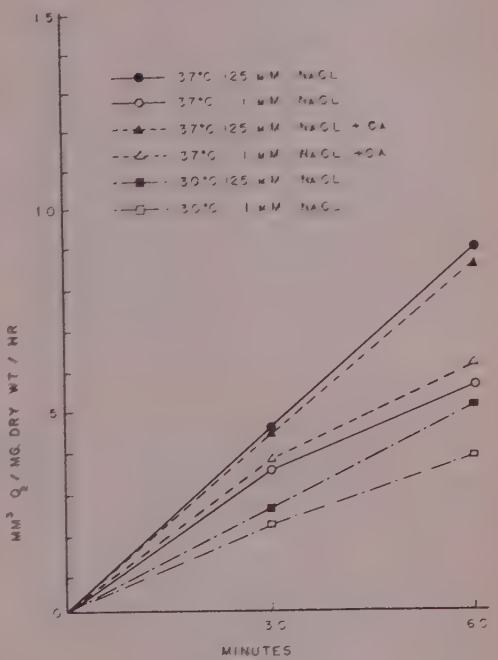

Fig. 1.

Effects of varying the temperature and the concentration of sodium chloride and calcium on the oxygen consumption of excised fish gills.

* We wish to make grateful acknowledgement to Dr. Fern W. Smith, who supervised the fixing and staining of the tissues, and aided in the interpretation of the sections.

1. McNemar, Q., Psychological Statistics, N. Y., Wiley, 1949.

Effects of lowered temperature. The above experiment was repeated at the temperature of 30°C. At this temperature the oxygen consumption was 37.5% less in the 125 mM solution, and 20% less in the 1 mM sodium chloride than was found at 37°C. The oxygen utilization is nearly linear with time in both instances (Fig. 1). The 60 minute oxygen consumption per mg of dry wt was 5.1 ± 0.21 mm^3 (mean of 16 fish) for the stronger solution, compared to 3.8 ± 0.22 mm^3 (mean of 16 fish) for the more dilute solution. The fact that the oxygen consumption did not fall as much in the weak solution as it did in the 125 mM solution with decrease in temperature was interpreted as indicating less disintegration of the tissue in the 1 mM solution at 30°C. This interpretation is consistent with the effect of temperature on the adhesiveness of mammalian cells reported by Zeidman(2).

Effect of calcium ions. Two series of gills were run, one in 125 mM and one in 1 mM sodium chloride. Each solution contained 2.75 millimoles of calcium ion per liter, added as calcium chloride. The determinations were made at 37°C. The mean oxygen consumption for 8 fish in the 125 mM solution was 8.4 ± 0.33 mm^3 O_2/mg dry wt/hr and the mean for an equal number of fish in the 1 mM solution was 6.1 ± 0.41 mm^3 O_2/mg dry wt/hr. Statistically, the effect of calcium on the oxygen consumption was not significant. The gross appearance and histological picture of the tissue were not improved by the presence of calcium.

Effects of rutin.† Rutin was added to the medium surrounding the gills to determine whether it would have a protective action against the deterioration of the gill tissue in 1 mM sodium chloride at 37°C. The right gills of 6 fish were placed in 1 mM sodium chloride, which in addition to the glucose added as substrate, was saturated with rutin. The left gills were in 1 mM sodium chloride without rutin. The mean oxygen consumption for the left gills was 4.7 ± 0.27 mm^3 O_2/mg dry wt at the end of 30 minutes and 7.7 ± 0.37 mm^3 O_2/mg dry wt at the end of 60 minutes; and for the gills in the rutin solution 4.5 ± 0.31 mm^3 O_2/mg dry wt at 30 minutes and 7.7 ± 0.44 mm^3 O_2/mg dry wt at the end of one hour. This was not a significant difference nor was the oxygen consumption as great during the last 30 minutes as during the first 30 minutes.

This set of experiments was initiated 9 months after the previous series had been completed. The fish used in the experiments with rutin came from the hatchery ponds in the late Fall and were better nourished than the other fish which were obtained in the late Winter. This probably accounts for the high Qo_2 values obtained. Even so the gross appearance and histological picture was not improved by the presence of rutin.

Discussion. The histological observations point to a sloughing of cells from the gill filaments in 1 mM sodium chloride possibly as a result of failure of the intercellular cement. It is not clear whether this effect is directly on the cementing substances or secondary to an effect on the living protoplasm of the cell. Presence of free nuclei indicated that some cells had broken down and lost their cytoplasm. The depressed oxygen consumption in this strength of solution can be the result of several factors: In part it may follow from the breakdown of tissues and cells with the loss of what Bodine(3) has termed "respiration of structure", and in part from reduction of oxygen uptake following the dilution of enzymes and substrates from the broken cells by the suspension medium.

Most capillary beds are subjected to an electrolyte concentration that is not only the same on both sides of the cellular layers, but is also isotonic to the cells. The gills of fresh water fishes however are bathed with plasma on one side and a very dilute solution on the other. The gill of the intact fish, unlike the excised gill, has the ability to maintain its structure, either by constant repair of the tissue, or by factors, as yet unknown, in the blood stream. The presence of ample oxygen

2. Zeidman, I., *Science,* 1949, v109, 596.

† The rutin, a quercetin rhamnoglucoside, was of lot 47-176 from the Eastern Regional Research Laboratory, Philadelphia, U. S. D. A.

3. Bodine, J. H., *Science,* 1950, v112, 110.

and glucose substrate are not in themselves sufficient for normal metabolism of excised gills in 1 mM sodium chloride. While calcium ions are reported to increase cell adhesiveness (4) addition of amounts equivalent to that found in blood did not materially increase O_2 consumption.

The results obtained with rutin tend to confirm the current opinion(5) that this substance has no particular influence on the maintenance of the intercellular cement.

The increased tolerance to dilute solutions exhibited by excised gills at 30°C over that found at 37°C adds support to the contention of Zeidman(2) that increased cellular adhesion with lower temperatures is a factor of importance to poikilothermic animals. This seems to be true, at least for fresh-water forms.

Summary. Excised gills of goldfish cannot maintain a respiratory rate as high in 1 mM sodium chloride as gills in 125 mM sodium chloride at 37°C even though the intact fish can live indefinitely in these solutions. Studies on the oxygen consumption of gill tissue supplemented with histological observations, indicate that the gill is protected not only by increasing the sodium chloride concentration to 125 millimolar, but by reducing the temperature to 30°C. However, the tissue is not significantly benefitted by the addition of either calcium or rutin to 1 mM sodium chloride.

4. Hober, R., Physical Chemistry of Cells and Tissues, Philadelphia, Blakiston, 1945.

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Received January 3, 1951. P.S.E.B.M., 1951, v76.

Passive Immunity in Poliomyelitis: II Lansing Antibody Content of Human Gamma Globulins.* (18496)

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Work is being carried out in these laboratories on some of the problems of passive immunity in experimental poliomyelitis, for which the Lansing strain of virus serves as a convenient model. On the one hand, attempts are being made to produce in monkeys, horses, and rabbits large quantities of high titer antiserum that can be subjected to fractionation. A report on one aspect of this part of the study has been prepared, describing the production of immune serum in the horse(1). On the other hand, search is also being made for a potent source of Lansing antibody from human sources, for it has been known for some years that gamma globulin prepared from pools of human plasma contains this antibody, as demonstrated by virus neutralization or passive protection experiments in mice(2,3). More recently, Bodian (4), carrying out virus neutralization tests in monkeys, has extended these earlier observations and shown that plasma gamma globulin contains antibodies for not only the Lansing, but also the Brunhilde and Leon types, in high titer. He found, for example, that gamma globulin diluted 1:100 neutralized 100 PD_{50} of virus.

This paper describes virus neutralization tests and passive protection experiments carried out in mice, with plasma as well as placental gamma globulin of human origin.

Materials. The placental globulin (Lederle) was purchased commercially. This product contains at least 16% gamma globulin and is sold for the prevention or modification of measles. The plasma gamma globulin was fur-

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Rhodes, A. J., Clark, E. M., and Shimada, F. T., *Canad. J. Publ. Hlth.*, 1951, v42, 23.

2. Enders, J. F., *J. Clin. Invest.*, 1944, v23, 510.

3. Stokes, J., Jr., *Yale J. Biol. Med.*, 1944, v16, 415.

4. Bodian, D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 259.

nished by the American National Red Cross. This product likewise contains at least 16% gamma globulin and is recommended for measles prophylaxis. Both of the gamma globulin preparations contained 1:10,000 merthiolate.

Methods. Virus neutralization tests. The neutralization test that we have used is similar to that employed in the studies of Morgan(5), and the technical details have been fully described(1). Briefly, serial 10-fold dilutions of gamma globulin were prepared in normal saline. To these dilutions were then added equal volumes of Lansing virus mouse pool. The pool was used in various dilutions, so that the final amounts of virus in the mixtures inoculated in mice varied from 8 to 85 LD_{50}. After one hour of contact at room temperature, groups of 10 mice (males 14-16 g weight) were inoculated cerebrally with 0.03 ml quantities of the virus-globulin mixtures. The mice were observed for 5 weeks and all deaths occurring after the day following inoculation were regarded as specific. The 50% neutralizing capacity of the globulin was then calculated by plotting the probits of the percentage mortalities in the groups of mice against the globulin dilutions in logarithms, drawing a straight line and reading off the globulin dilution where 50% mortality might be expected. It should be realized that the dilutions of globulin mentioned in this paper refer to the dilutions before admixture with an equal volume of virus.

Passive protection experiments. In these experiments, various materials containing Lansing antibody, along with suitable controls, were inoculated peritoneally (0.3 ml) in 14-16 g male mice. Twenty-four hours later the mice were challenged by the cerebral route with 5 or 25 LD_{50} of Lansing virus, and thereafter were observed for 5 weeks.

Results. Virus neutralization tests. The results of 16 titrations of lot No. 2167 of placental gamma globulin on 11 different working days are given in Table I. The average 50% neutralizing titer was $10^{-3.5}$, the lowest figure in any titration being $10^{-2.5}$ and the highest $10^{-4.0}$. These differences are probably not greater than can be explained by the operation of chance, having regard to the rather widely spaced dilutions of globulin used. It will be apparent that the neutralizing titer was little affected by the amount of virus added to the virus-globulin mixtures, as shown for instance in the tests performed on 26 October, 1950. It has also been demonstrated that heating gamma globulin to 56°C for 30 minutes does not lower the antibody titer (Experiment of 1 November, 1950).

The 7 lots of plasma gamma globulin were prepared from very large pools of human plasma, each representing at least 20,000

TABLE I. Estimation of 50% Neutralizing Titers of Placental Gamma Globulin.

Date of test	Reference No. of sample tested	Amt of virus in neutralization tests (in LD_{50})	50% neutralizing titer
21 Sept. '50	2167-56A	85	$10^{-2.5}$
29 "	"	85	$10^{-4.0}$
3 Oct.	"	85	$10^{-3.75}$
18 "	"	85	$10^{-3.35}$
20 "	2167-86A	85	$10^{-2.8}$
20 "	"	17	$10^{-3.46}$
24 "	"	17	$10^{-3.65}$
26 "	"	85	$10^{-3.53}$
26 "	"	25	$10^{-3.0}$
26 "	"	12	$10^{-3.75}$
26 "	"	8	$10^{-3.5}$
1 Nov.	" (heated 56°C ½ hr)	17	$10^{-3.75}$
1 Nov.	2167-86A (unheated)	17	$10^{-3.75}$
2 "	2167-86A	17	$10^{-3.75}$
8 "	"	17	$10^{-3.55}$
10 "	"	17	$10^{-3.82}$

TABLE II. Lansing Virus Neutralization Antibody Titers of 7 Samples of Human Gamma Globulin (Plasma).

Globulin Lot No.	Amt of virus in neutralization tests (in LD_{50})	50% neutralizing titer of globulin
113-1	21	$10^{-3.0}$
116-4	21	$10^{-3.5}$
12OR-3	21	$10^{-3.65}$
121-1	21	$10^{-3.27}$
127-2	21	$10^{-4.0}$
128-2	21	$10^{-3.0}$
129-2	21	$10^{-2.87}$

5. Morgan, I. M., *Am. J. Hyg.*, 1947, v45, 372.

TABLE III. Passive Protection Experiment in Mice.

Inoculum administered (0.3 ml peritoneally 24 hr before virus)	50% neutralizing titer of material inoculated	Mortality in mice challenged cerebrally with following doses of virus	
		25 LD_{50}	5 LD_{50}
Placental gamma globulin	$10^{-3.5}$	8/20 (40%)	4/19 (21.2%)
Immune monkey serum	$10^{-2.5}$	12/20 (60%)	7/20 (35 %)
Immune horse serum	$10^{-2.5}$	18/19 (95%)	13/20 (65 %)
Normal monkey serum	Nil	19/19 (100%)	15/19 (79 %)
Normal horse serum	Nil	19/19 (100%)	19/19 (100 %)

TABLE IV. Passive Protection Experiment in Mice.

Inoculum administered (0.3 ml peritoneally 24 hr before virus)	50% neutralizing titer of material inoculated	Mortality in mice challenged cerebrally with following doses of virus	
		25 LD_{50}	5 LD_{50}
Plasma gamma globulin	$10^{-2.63}$	10/18 (55.6%)	5/19 (26.3%)
Placental gamma globulin	$10^{-3.5}$	8/18 (44.5%)	5/20 (25 %)
Saline		19/20 (95 %)	17/19 (89.5%)

donors. The 50% neutralizing capacities are shown in Table II, the titers ranging from $10^{-2.87}$ to $10^{-4.0}$. It is not considered that the observed differences in titer are significant. The antibody content would appear to be similar to that of placental globulin.

Passive protection experiments. Following some preliminary experiments, two rather detailed experiments have been performed in which the protective action of various sera and globulins containing Lansing antibody has been demonstrated. These preparations were inoculated in groups of twenty 14-16 g male mice by the peritoneal route (0.3 ml), and 24 hours later the animals were challenged with an estimated 25 or 5 LD_{50} of Lansing virus. The results of the first experiment are shown in Table III, from which it will be seen that when compared with controls, placental gamma globulin exerted a significant sparing effect against challenge with 5 LD_{50} and even 25 LD_{50} of virus. These results are similar to those recorded by Kramer(6) using human convalescent serum. It will also be noted that some sparing effect was shown by an immune monkey serum.

In the second experiment, plasma gamma globulin (lot No. 116) was included and compared with placental gamma globulin. The results are shown in Table IV from which it is evident that both products give significant protection against death in mice inoculated with 25 and 5 LD_{50} of virus.

Conclusions. Both placental and plasma gamma globulins contain Lansing antibody. The 50% neutralizing titer of these products is approximately $10^{-3.5}$, as determined in mice. It would seem that a more potent gamma globulin could be obtained if prospective donors were first tested for serum Lansing antibody level. Then only those with high levels would be used to furnish plasma for the pools to be fractionated. Placental and plasma gamma globulins have a significant sparing effect when inoculated in mice 24 hours before a cerebral challenge of Lansing virus.

Our thanks are due to Dr. G. Foard McGinnes and Dr. Charles A. Janeway of the American National Red Cross, and to Dr. John W. Palmer of E. R. Squibb & Sons, who made arrangements for us to receive samples of plasma gamma globulin for test.

6. Kramer, S. D., *J. Immunol.*, 1943, v47, 67.

Received January 25, 1951. P.S.E.B.M., 1951, v76.

Prevention of Toxicity of Massive Doses of A-methopterin by Citrovorum Factor.* (18497)

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It has been demonstrated that citrovorum factor (CF) is more effective than pteroylglutamic acid (PGA) in preventing the inhibition of growth of *Leuconostoc citrovorum* by 4-amino-PGA (aminopterin)(1-4). CF has also been shown to prevent the toxic effects of aminopterin in mice(5) and in rats (4), and to be more active on a dry weight basis than PGA in preventing the chemotherapeutic effect of 4-amino-N^{10}-methyl-PGA (A-methopterin) in a strain of mouse leukemia(6,7). Studies by Franklin *et al.* indicate that PGA can prevent the toxicity of aminopterin(8) and A-methopterin(9) only when the antagonists are administered at ranges near the minimum lethal dose. At higher doses, PGA is ineffective. Since earlier investigation has demonstrated that CF is effective at somewhat higher levels of aminopterin(5) than is PGA, it was considered worthwhile to study the effects of CF‡ on the toxicity of high acute and chronic dosage of A-methopterin.‡

Method. Young mice of the Ak stock weighing 17-23 grams and fed the usual diet of Purina Laboratory Chow were used in these experiments. Injections of CF and A-methopterin were given by the intraperitoneal route either once in the acute experiments, or daily for 5 days in the chronic studies. The mice were observed for two weeks after the administration of the first dose of A-methopterin. The results of the studies can be seen in Tables I and II.

Results and discussion. Studies in mice by Ferguson, Thiersch, and Philips(10) have shown the acute LD_{50} for A-methopterin to be 94 mg/kg and the chronic LD_{50} when given daily for 5 doses to be 1.9 mg/kg.

The results in Table I demonstrate that considerable protection against the acute lethal effects of a single dose of 250 mg/kg of A-methopterin may be achieved by as little as one thirtieth that amount of CF given either one hour before or after the dose of antimetabolite. Almost complete protection from this dose of A-methopterin was afforded by doubling the CF to 15 mg/kg. When the CF was given 24 or 48 hours before the A-methopterin, however, no protective effect was noted. There was also little or no significant protection when the CF was administered 4 hours or more after the anti-

* This investigation was supported, in part, by a research grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, in part by an institutional grant from the American Cancer Society, and, in part, by funds from the Damon Runyon Memorial Fund for Cancer Research, Inc. and the Babe Ruth Memorial Fund.

† Senior Fellow of the Damon Runyon Memorial Fund for Cancer Research.

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9. Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. B. C.*, 1949, v177, 621.

‡ We wish to acknowledge the generosity of the Lederle Laboratories Division of American Cyanamid Company in supplying these compounds.

10. Ferguson, F. C., Jr., Thiersch, J. B., and Philips, F. S., *J. Pharm. and Exp. Therap.*, 1950, v98, 293.

TABLE I. Effect of Single Acute Doses of A-Methopterin and Citrovorum Factor.

Dosage in mg/kg A-meth.	CF	CF inj. in relation to A-meth.	Mortality	Day of death 1	2	3	4	5	6	7	8	9
125			10/10			6			4			
250			14/15			5	6		2		1	
250	1.9	15 min. before A-methopterin	7/10			4	2		1			
250	3.8	" " " "	9/10			6	3					
250	7.5	" " " "	5/10			1	1		3			
250	15	" " " "	2/10						2			
250	30	" " " "	1/15						1			
250	30	1 hr " "	3/10				2	1				
250	30	24 " " "	8/10			5	1	2				
250	30	48 " " "	9/10				3		5			1
250	3.8	1 " after "	6/10						6			
250	7.5	1 " " "	5/10			1			4			
250	15	1 " " "	0/10									
250	30	1 " " "	6/14			1			5			
250	30	2 " " "	3/5				2		1			
250	30	4 " " "	3/4			2			1			
250	30	6 " " "	5/5			1	3		1			
250	30	25 " " "	5/5			2	2		1			

TABLE II. Effect of Daily Doses(5) of A-Methopterin and Citrovorum Factor.

Dosage in mg/kg A-meth.	CF	CF inj. in relation to A-meth.	Mortality	Day of death 1	2	3	4	5	6	7	8	9
10	—	—	19/19			6	9	2		2		
10	0.75	15 min. before A-methopterin	1/10								1	
10	1.5	" " " "	0/10									
10	3	" " " "	0/10									
50	—	—	29/29			14	10	2		3		
50	7.5	15 min. before A-methopterin	4/10				1			3		
50	30	1 hr before 1st and 15 min. before all inj. of A-methopterin	0/20									
100	7.5	15 min. before A-methopterin	10/10				1			9		
100	30	1 hr before 1st and 15 min. before all inj. of A-methopterin	0/10									
200	7.5	15 min. before A-methopterin	10/10				2	1		7		
200	30	1 hr before 1st and 15 min. before all inj. of A-methopterin	4/10					2				2
500	7.5	15 min. before A-methopterin	10/10				1			9		

metabolite.

When the CF was given just prior to the A-methopterin (Table II) and a schedule of 5 daily injections was employed, the toxicity of 10 mg/kg (5 x LD_{50}) was almost completely prevented by 0.75 mg/kg of CF. At 50 mg/kg of A-methopterin 7.5 mg/kg of CF, and at 200 mg/kg of A-methopterin, 30 mg/kg of CF protected 60 per cent of the mice.

The clinical effectiveness of simultaneous administration of large doses of CF in *preventing* the toxic mouth ulcerations caused by aminopterin and A-methopterin has been reported(11). The diminished protective action of CF when it was given to mice 4 hours after A-methopterin, however, suggests that CF would not be of great usefulness in *reversing* the lesions of A-methopterin toxicity once they have occurred and the antagonist has been discontinued. Also since it has previously been shown that CF prevents the antileukemic effects of A-methopterin in mice(6) as well as the toxic effects, it is doubtful whether it has any practical usefulness in the therapy of acute leukemia in children.

The fact that the A-methopterin/CF ratio changed relatively little in these chronic studies up to 100 times the LD_{50} of A-methopterin indicates a competitive type of relationship between these two compounds.

11. Schoenbach, E. B., Greenspan, E. M., and Colsky, J., *J.A.M.A.*, 1950, v144, 1558.

This is in marked contrast to the inability of PGA to protect against much smaller doses of A-methopterin(9).

Thus, from these studies and those of others it would appear that aminopterin and A-methopterin are true antagonists of citrovorum factor rather than of folic acid.

Summary. 1. The administration of citrovorum factor 15 minutes prior to seven to thirty times as large a dose of A-methopterin, prevented the toxicity of the antimetabolite at levels ranging from 2 to 100 times the chronic LD_{50}. 2. No significant protective effect was noted when similar dosages of citrovorum factor were given four or more hours after the administration of A-methopterin. 3. A-methopterin appears to be a true antagonist of citrovorum factor rather than of pteroylglutamic acid.

Received January 18, 1951. P.S.E.B.M., 1951, v76.

Role of Aureomycin and Citrovorum Factor in "Folic Acid" Deficiencies.* (18498)

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The use of folic acid antagonists in the treatment of acute leukemia in children has demonstrated that aminopterin and a-methopterin prolong life and give satisfactory clinical improvement in many cases. It has never been adequately explained why some cases similarly managed appear to be entirely unaffected by antagonist therapy. In several laboratory animals the antagonist is toxic to normal bone marrow and produces a leukopenia, growth retardation and hemorrhagic phenomena. No single metabolic action has as yet been suggested to account for these multiple findings. The consideration that more than pteroylglutamic acid (PGA) is antagonized by aminopterin finds some support in the inability of relatively large amounts of this vitamin to overcome small dosages of aminopterin. Our experiments to elucidate the mechanism of action of antifolic acid compounds were designed to test the possibility that more than PGA alone is affected by the antagonists. To obtain information on other factors that may influence the production of a PGA deficiency, it seemed necessary to compare the deficiencies induced by different means. At least three experimental approaches are available to obtain "folic acid" deficiencies: (1) The omission of PGA from a complete diet, (2) The use of PGA antagonists, and (3) The addition of relatively insoluble sulfonamide drugs to the diet. Since the deficiency produced by mere omission of the vitamin from the diet is time consuming and variable, this method was not used. The experiments described below provide evidence that the latter 2 methods may induce other deficiencies in addition to the now recognized PGA deficiency. Accordingly, the term "folic acid" deficiency will be used in the body of the paper to denote the condition observed on the sulfa diet or on the diet containing the antagonist. Since these diets probably produce a multiple rather than a single deficiency, reports by previous workers should be re-evaluated.

Experimental. Sprague-Dawley rats, 24 days of age, were given a synthetic diet consisting of sucrose, 73 parts; vitamin-free casein, 18 parts; salt mixture,† 4 parts; thiamine, 0.5 mg; riboflavin, 0.5 mg; niacin, 1 mg; calcium pantothenate, 2 mg; pyridoxine, 0.5 mg; inositol, 100 mg; biotin, 0.002 mg; and choline, 300 mg; vitamin B_{12}, .010 mg. Two drops of oleum percomorphum was given by mouth twice weekly to each rat as a

* Supported in part by a grant from the United States Public Health Service.

† Salts No. 2, Nutritional Biochemicals, Chagrin Falls, Ohio.

source of vitamins A and D. When 2% sulfaguanidine or 2% sulfaphthalidine was included in the synthetic basal diet, the sucrose was proportionately reduced.

Results. Aminopterin-induced "folic acid" deficiency. It had already been established by others(1) that 50-100 μg of aminopterin per 100 grams of diet produces poor weight gain, leukopenia, hemorrhage and death in a period of 3 to 4 weeks. In our experiments, however, despite the apparent uniformity of animals from a commercial source, the variability of response by the individual rats to the antagonist made adequate controls imperative. Data were obtained in several series to confirm the fact that aureomycin gives better growth than a diet without the antibiotic. The effect of the antagonist was tested, therefore, in a diet which included aureomycin. Adult rats previously fed the basal diet continued to thrive when given 75 μg of aminopterin plus 5 mg of aureomycin per 100 g of diet. Other adult rats given both 5 μg aminopterin and 5 mg of aureomycin as a single daily intraperitoneal injection for 14 days showed little change in weight, again demonstrating surprising protection by aureomycin against toxic levels of aminopterin. Because Weintraub *et al.*(2) and Goldin *et al.*(3) found that male mice are more susceptible than female mice to toxic levels of aminopterin, it seemed desirable to substantiate our findings under these more rigorous experimental circumstances by using only male weanling rats. A typical series

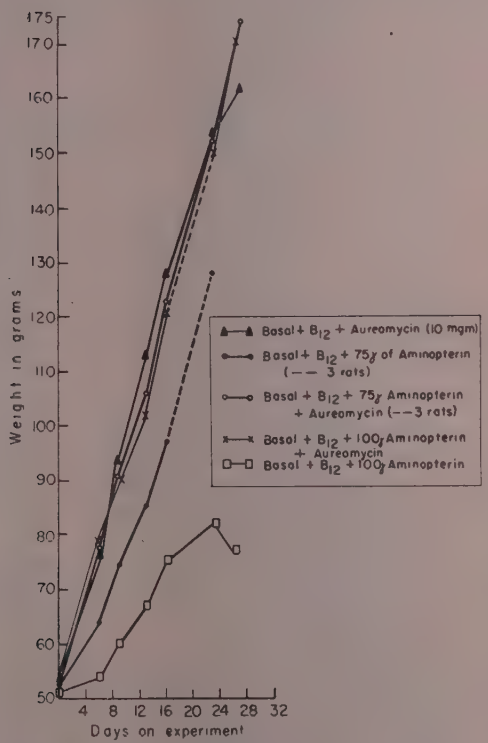

FIG. 1.

Effectiveness of aureomycin in overcoming the aminopterin induced "folic acid" deficiency.

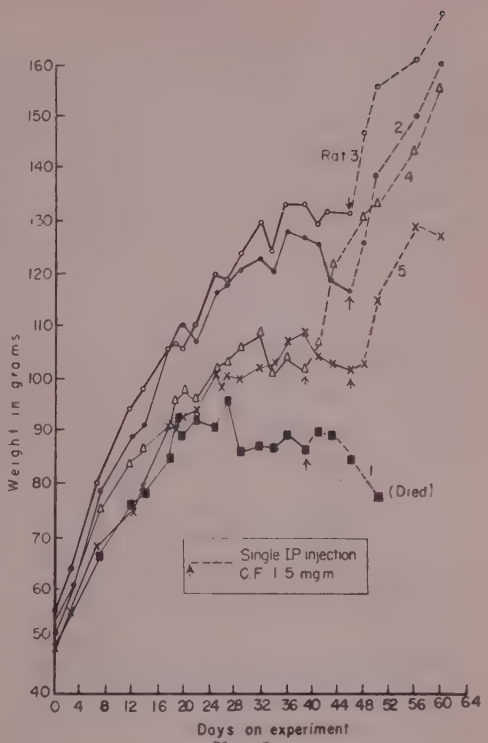

FIG. 2.

Typical growth response following a single intraperitoneal injection of citrovorum factor concentrate to individual rats fed the sulfaphthalidine diet.

1. Oleson, J. J., Hutchings, B. L., and SubBarrow, Y., *J. Biol. Chem.*, 1948, v175, 359.

2. Weintraub, S., Krause, S. D., and Wright, L. T., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 609.

3. Goldin, A., Greenspan, E. M., Goldberg, B., and Schoenbach, E. B., *Cancer*, 1950, v3, 849.

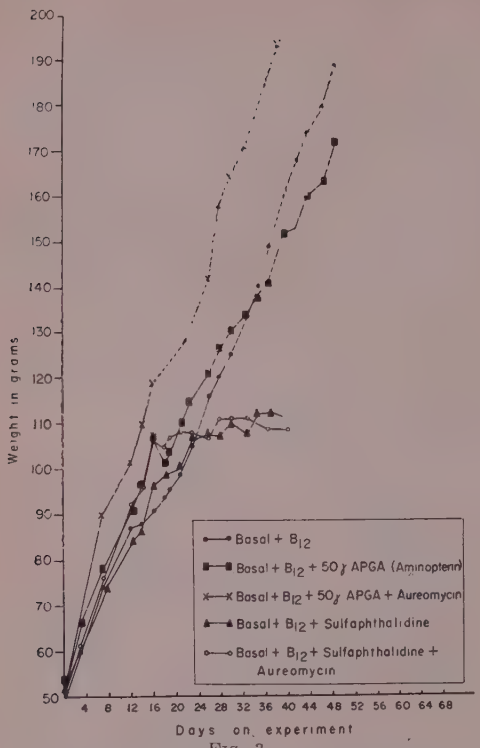

FIG. 3.

Ineffectiveness of aureomycin in group receiving sulfa diet in contrast to good growth response in group receiving minimal level of aminopterin.

contained groups in which 5 or 10 rats were given 50, 75 or 100 μg of aminopterin per 100 g of diet. Growth on 50 μg of aminopterin was slightly below that of controls not receiving the antagonist. The increasing toxicity of the 75 and 100 μg level in the diet was evidenced by the fact that 2 rats succumbed on the 75 μg diet, and all died on the 100 μg level. Fig. 1, 3 and 4 give graphic evidence that the beneficial effect of aureomycin occurs with 50 μg, 75 μg or 100 μg of aminopterin in the diet. It will be seen that while aminopterin control rats fail before 4 weeks (Fig. 1), the aureomycin plus aminopterin groups give as good growth as the control diet containing aureomycin but without aminopterin. Two rats which did not eat the aminopterin plus aureomycin diet well had poor weight gain; it was of interest, therefore, that these animals showed significant improvement in weight when 5 mg of aureomycin was given intraperitoneally. We have used 65 rats on aureomycin diets and twice this number on suitable control diets to confirm these observations.

The question whether aureomycin is a growth factor *per se* or whether its growth promoting properties are the result of antibiotic action cannot be completely answered at this time. It is known that autoclaving aureomycin at an alkaline pH destroys the antibiotic activity. Fig. 4 not only shows additional data on the ability of aureomycin to counteract aminopterin toxicity, but also provides evidence that alkaline autoclaving destroys the beneficial effect of the antibiotic on growth.

Sulfa-induced "folic acid" deficiency. It has always been presumed that PGA formed by intestinal bacteria is subsequently absorbed for purposes of growth in the host and that these bacteria are inhibited by an in-

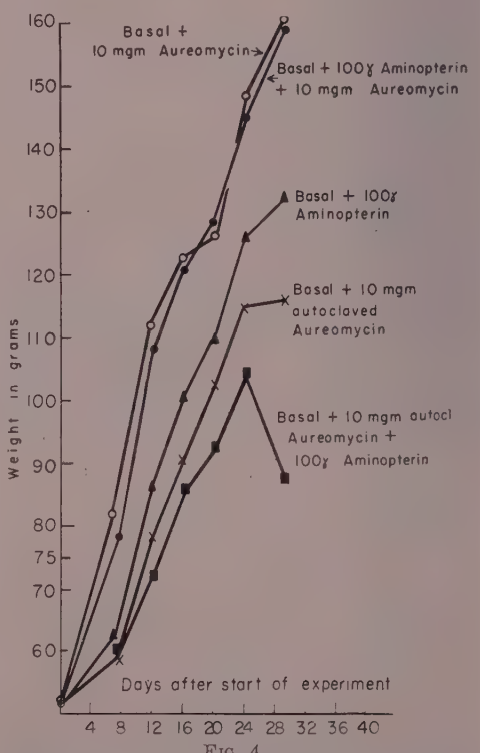

FIG. 4.

Ineffectiveness of autoclaved aureomycin in promoting growth or counteracting aminopterin toxicity.

soluble sulfonamide. The addition of PGA alone, however, does not provide optimum growth in rats on diets containing such sulfa drugs, and inhibition of intestinal flora may limit not only PGA but other growth factors required by the rat. Our data clearly show the effectiveness of concentrates of citrovorum factor‡ in correcting the "folic acid" deficiency. Fig. 2 shows the typical gain in growth that results when 10×10^6 citrovorum factor units§ are given as a single injection to individual rats fed the sulfaphthalidine diets. These responses were seen in more than 25 animals after they were maintained for 36-48 days on the sulfaphthalidine diet. In several instances (not illustrated) injection of the citrovorum concentrate into deficient animals was followed by a latent period before a manifest response. It is also of interest that in several rats citrovorum factor was ineffective once the rats had received single intraperitoneal injections of 200 μg of PGA.

The role of aureomycin as a growth stimulus has not been previously examined in rats receiving 2% sulfa diets. When 2.5 or 5.0 mg of aureomycin was either given intraperitoneally to the deficient animals or was added to 100 g of diet, increases in weight were not significantly higher than in the controls. A similar result was obtained when 10 mg of aureomycin was added to 100 g of diet containing 2% sulfaphthalidine from the start of the experiment (Fig. 3). This finding is important, especially since we were able to show that aureomycin could counteract the deleterious effect obtained on a diet which contained aminopterin.

Discussion. The present experiments have been primarily directed at elucidating the mode of action of the PGA antagonists. Although PGA is part of an important enzyme system necessary for growth or for cellular metabolic processes, recent work has indicated that citrovorum factor is perhaps the more active portion of that enzyme system. It was therefore not unexpected that citrovorum factor concentrates were more effective than PGA in correcting the "folic acid" deficiency produced either by aminopterin or sulfa drugs. It might be more exact, perhaps, to describe aminopterin as a citrovorum factor antagonist as well as a PGA antagonist. However, the fact that PGA is a precursor in the formation of citrovorum factor may indeed explain the effectiveness of aminopterin as an antagonist.

The most significant finding of the present report is the effectiveness of aureomycin in overcoming the toxic effects of aminopterin. The inability of toxic levels of aminopterin to exert their effect in the presence of aureomycin was apparent whether the aureomycin was given from the start or after the onset of the deficiency.

It is of more than passing interest that autoclaved aureomycin was not able to provide the growth response obtained with untreated antibiotic. The assumption can be made that since the antibiotic activity was destroyed, the mode of action of the compound is through its effect on intestinal flora. This may not be completely tenable, since if an additional growth factor exists in crystalline aureomycin, it too may be destroyed by the autoclaving. Further work on this point is necessary, but several possibilities may be entertained, none of which are as yet documented by adequate data: (a) It may be that the role of aureomycin is merely that of an antibiotic depressing the growth of those intestinal microorganisms that utilize the PGA produced by favorable bacteria, thus making the vitamin unavailable to the host. (b) Aureomycin may actually be a growth factor, and the findings here are unrelated to its antibiotic activity as such. All or part of the aureomycin molecule may function as a prosthetic group for an essential coenzyme. (c) Still another possibility is that aureomycin may interfere with those natural inhibitors of folic acid conversion to citrovorum factor. (d) A last possibility is that aureomycin has a structure essentially similar to that of PGA or citrovorum factor and can replace either in the diet. Experiments are in progress to determine whether any or all of these hypotheses have any credence.

‡ Kindly supplied by Dr. H. Broquist, Lederle Laboratories, Inc.

§ 10,000,000 units are 0.75 mg (Dr. H. Broquist).

Summary. 1. "Folic acid" deficiencies induced by aminopterin and by sulfa drugs in rats have been compared. 2. Aureomycin can counteract the toxic effects of 50, 75 and 100 μg of aminopterin/100 g of diet. The antibiotic is equally effective whether fed in the diet or given intraperitoneally. 3. Aureomycin is without effect on growth in rats made "folic acid deficient" on a sulfa diet. 4. Citrovorum factor concentrates are able to completely overcome the deficiency produced on either type of diet.

Received January 9, 1951. P.S.E.B.M., 1951, v76.

Complement-Fixing Murine Typhus Antibodies in Vitamin Deficiency States.* (18499)

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The effect of malnutrition in epidemic typhus fever has been recognized for some time. Efforts have been made to find an explanation for the observation that increased mortality occurs in malnourished populations exposed to typhus fever. FitzPatrick(1) injected weanling male white rats, subjected to various vitamin and protein deficiencies, with viable rickettsiae (*Rickettsia typhi*) of murine typhus fever. It was demonstrated that the animals on a reduced vitamin B complex intake of 1/10th the optimal level were more susceptible than those receiving ample B vitamins. Thiamin, riboflavin, and pantothenic acid deficiencies studied separately, caused increasing susceptibility. It was also reported that animals on a natural diet were more resistant than those on a complete synthetic ration. The purpose of this investigation was to extend these observations. It was felt that the increase in susceptibility might in some manner be correlated with the inability of the animals to rapidly produce circulating antibodies when antigenic material was introduced.

The production of circulating antibodies in vitamin deficient states has been reported by a number of investigators. The antigenic materials employed for these studies have been of a variety of types. Axelrod, Carter, McCoy and Geisinger(2) demonstrated that pyridoxin, pantothenic acid deficiencies effected the antibody production by the rat in response to the injection of human red blood cells as antigenic stimulus. Carter and Axelrod(3) reported that the content of circulating antibodies in the thiamin and biotin deficient rats was less than that of the control rats. Ludovici, Axelrod, and Carter(4) observed a low production of circulating antibodies in the pantothenic acid deficiency state when rats were injected with human red blood cells. Stoerk, Eisen, and John(5) reported that pantothenic acid deficiency did not affect the level of circulating antibodies when the rats were immunized with sheep erythrocytes. In the present study 5 different diets were utilized. In the initial investigation (Table I), 5 rats were placed on an adequate diet and 5 were placed on a diet containing only one-tenth of the amount of vit. B complex considered to be the optimum. The second experiment employed 4 groups of animals; these were total vit. B deficiency,

* This investigation was supported by a research grant from The National Institutes of Health, Public Health Service.

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5. Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.,* 1947, v85, 365.

TABLE I. C.F. Titers with Murine Typhus Rickettsiae and Rat Sera After Immunization with 4 cc Vaccine.

	No.	C.F. titers following immunization		
		1 wk	2 wk	3 wk
Normal control	1	1/640	1/640	1/320
Adequate B	2	1/320	1/320	*
Complex group	3	1/320	1/320	1/160
	4	1/320	1/320	1/160
	5	1/320	1/320	1/640
1/10th optimal	6	1/320	†	1/320
Level B complex	7	1/320	1/320	1/640
Group	8	1/640	†	1/640
	9	1/320	1/320	1/640
	10	1/320	1/320	1/640

All pre-sera were negative.
* Animal died following second bleeding.
† Insufficient serum.

pantothenic acid deficiency, thiamin deficiency, and an adequate vit. B complex control group. An effort was made to reproduce conditions comparable to those studied by FitzPatrick(1). The animals were subsequently immunized with washed, formalinized suspensions of *Rickettsia typhi* (murine typhus).† Following immunization, the rats were bled and the circulating antibodies were demonstrated by complement fixation(6-8).

Experimental. Ten male albino rats of the Sprague-Dawley strain, distributed into 2 groups as indicated in Table I, were housed individually in wide-meshed, screen-bottom cages and weighed daily. The basic diet was composed of 65.12% sucrose, 17.23% vitamin test casein, 9.55% vegetable oil hydrogenated, 3.83% General Biochemicals, Salt Mixture No. 2, 2% Squibb's Cod Liver Oil, 2% Mazola Corn Oil, 0.2% choline chloride, 0.03% i-inositol and 0.01% p-aminobenzoic acid. Rats on the adequate vit. B complex control group received their vitamins in the form of a daily pill, supplying the following vitamins: thiamin, 40 γ; pantothenic acid, 150 γ; pyridoxin, 50 γ; niacin, 100 γ; riboflavin, 60 γ(9). Rats on the one-tenth adequate B ration were given a pill daily which supplied one-tenth the amount of vitamin given to the normal control group. The basal diet was fed to the one-tenth adequate controls and the intake of each rat of the normal control group was restricted to that consumed during the previous day by the rats on the deficient diet.

At the end of the 6-week period, each rat was injected intraperitoneally with 1 cc of murine typhus vaccine. The inoculations were repeated at 7-day intervals until each rat had received a total of 4 cc. During this period, the animals were maintained on the same diet to that fed prior to immunization. The rats were bled 1, 2, and 3 weeks after the last inoculation of the rickettsial suspension. The sera were obtained under sterile conditions and stored at –20°C until tested. The complement-fixing antibodies produced by rats maintained on total vit. B, pantothenic acid, and thiamin deficiencies are indicated in Table II. These animals were bled 7 days after each inoculation of murine typhus vaccine to determine if the amount of antigenic stimulus played a role. These animals were maintained as were those recorded in Table I with the exception of their diets and of the time of bleeding. The complement fixation technic was employed for measuring the circulating antibody response. The technic employed in this study was identical to that of Plotz(6), Plotz and Wertman(7), and Plotz, Reagan, and Wertman(8). Fixation was allowed to take place for 18 hours at 4 to 6°C. The sensitized cells (0.25 ml sheep cells 3% and 0.25 ml amboceptor containing 3 MHD) were then added and the tubes incubated for 30 minutes at 37°C in a waterbath. The tests were read following secondary incubation and only 4+ and 3+ fixation were accepted as end-points. The controls necessary to insure valid results were included in all the titrations and serum dilution tests.

Results. The normal control rats at the end of the 6-week feeding period, all had

† Purified antigen supplied by Dr. Herald Cox, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

6. Plotz, H., *Science,* 1943, v97, 20.

7. Plotz, H., and Wertman, K., *Science,* 1942, v95, 441.

8. Plotz, H., Reagan, R., and Wertman, K., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 173.

9. Griffith, J. Q., and Farris, E. J., The Rat in Laboratory Investigations, Philadelphia, J. B. Lippincott, 1949, 2nd Ed.

TABLE II. Complement-fixation Titers and Body Weight Change of Rats in Vitamin Deficiency States.

Diet	No.	Complement-fixation titers following 1 inj.	3 inj.	Body wt g Init. avg, g	Final avg, g
Adequate Vit. B	1	1:40	1:1280		
	2	1:80	1:2560		
	3	1:20	1:1280	74	118
	4	Neg.	1:2560		
	5	1:20	—		
Total vit. B deficient	11	Neg.	—		
	12	1:20	—		
	13	1:20	1:320		
	14	1:20	1:1280	70	58
	15	1:10	1:640		
	16	1:20	1:320		
	17	1:10	1:640		
	18	1:10	1:320		
Pantothenic acid deficient	21	Neg.	1:320		
	22	Neg.	1:640		
	23	Neg.	1:1280		
	24	Neg.	1:320	68	102
	25	Neg.	1:1280		
	26	1:20	1:2560		
	27	1:20	—		
	28	1:20	1:2560		
Thiamin deficient	31	1:20	—		
	32	Neg.	1:160		
	33	1:40	1:640		
	34	1:20	1:640	69	68
	35	Neg.	1:320		
	36	Neg.	1:160		
	37	1:80	1:160		
	38	Neg.	1:320		

— Animals died during investigation.

gained weight, appeared healthy and possessed smooth even coats. The rats fed a diet containing only one-tenth of the optimum B complex content appeared to be in relatively poor condition. The normal control rats at the end of the 6-week period showed an average gain in weight of 128 g but the rats on the deficient diet showed an average gain in weight of only 36 g.

The rats used in the second experiment showed the usual signs which might be expected to accompany their respective deficiency. The initial average and final average weight of each group are recorded in Table II. The total deficient and thiamin deficient animals either lost weight or gained very little and the normal control and pantothenic acid deficient rats all showed gains in weight.

Table I demonstrates the end-point titers obtained in the complement fixation test using a washed suspension of murine typhus rickettsiae as the antigen. It appears that the ration containing one-tenth of the optimal level of vit. B complex did not seriously interfere with circulating antibody production when a relatively large amount of antigenic material was injected over a period of 4 weeks.

Table II demonstrates the end-point titers obtained in the complement fixation test using a murine typhus rickettsial antigen. It appears that the animals maintained on a pantothenic acid and thiamin deficient diet did not produce antibodies to the same extent as did those on a totally vit. B deficient diet, or those of the normal control group after the injection of only 1 cc of murine typhus vaccine. However, the sera specimens obtained after the administration of 3 cc of vaccine did contain demonstrable concentrations of complement-fixing antibodies. The animals were bled prior to immunization and tested by complement fixation. All of these sera were found to be negative with the rickettsial antigen used in this investigation.

Summary. These studies indicate that rats on a diet containing what is considered to be an optimum vit. B complex content have the ability to produce circulating complement fixing antibodies when immunized with relatively large amounts of the rickettsiae of murine typhus fever. Likewise, rats maintained on a diet containing only one-tenth of the presumed optimum also have the ability to produce these same antibodies to approximately the same titers. Pantothenic acid and thiamin deficiencies influenced the antibody response when a relatively small amount of immunizing material was injected. The influence is not as apparent when relatively large amounts of the antigenic material was employed.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Potassium Chloride Flooding and DCA-induced Cardio-renal Hypertrophy in the Rat. (18500)

Habeeb Bacchus. (Introduced by C. E. Leese.)
(With the technical assistance of C. A. Toompas and M. H. Heiffer).
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Desoxycorticosterone acetate (DCA) overdosage under suitable conditions induces cardiac and renal hypertrophy(1,2), adrenal atrophy(3), and periarteritis nodosa(1,4). Simultaneous treatment of rats on DCA-overdose with certain "acidifying" salts prevents these signs of cardiovascular damage, whereas similar treatment with potassium chloride does not(4). Similar KCl treatment to DCA-treated rats does not prevent the hypertension (5), or renal hypertrophy following DCA-overdosage(6). Subsequent work indicated that a dietary excess of potassium chloride leads to a decrease of cardiac mass, an increase of renal mass, and signs of adrenal glomerular zone hyperactivity; these changes are reversible(7).

This paper reports data which indicate that dietary excess of KCl to DCA-treated rats prevents the cardiac and adrenal changes characteristic of the overdosage, without altering the effect on the kidney.

Materials and methods. Four groups of female Wistar rats were treated as follows: Group I rats received single subcutaneous implants of 50 mg DCA, (Percorten, crystalline, Ciba), in gelatin capsules and were maintained on Purina Laboratory Chow and a 0.2% NaCl solution as the sole source of fluid. The NaCl was added in order to sensitize the animals to the effects of DCA; Group II rats received similar implants of DCA and were maintained as Group I rats except that their fluid supply contained 0.2% NaCl and 2% KCl. Group III rats were given implants of empty gelatin capsules and were maintained as those of Group II. Rats of Group IV served as controls and were kept on Chow and tap water (or 0.2% NaCl). Four of this group were given 0.2% NaCl; this solution produced no discernible effects on the animals. All animals were killed at the end of 30-32 days. The heart and kidneys were weighed; the hearts were kept for histological studies. One adrenal from each animal was used for the histochemical detection of cholesterol, the other for ascorbic acid, after methods used in another study(3).

Results. The final body weights of the 4 groups of rats were similar (200 g), therefore the absolute organ weights are presented. The mean heart weight of DCA-treated (Group I) rats is significantly greater, whereas the mean heart weight of KCl-treated (Group III) rats is significantly lower, than that of control animals. The mean heart weight of rats receiving DCA-KCl (Group II) is not different from control heart weight. Comparison of the observed heart weights with heart weights predicted from standard data(8) shows a similar pattern, that is, DCA increases, KCl decreases, heart weight, while DCA-KCl does not alter heart weight from normal range (Table I). Rats of groups I and II show kidney weights significantly greater than those of control animals. In this study as in a previous one, the observed kidney weights are much higher than the predicted weights. KCl was reported in a previous paper to induce an increase of renal mass(7).

The increase of heart weight observed in

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7. Bacchus, H., and Toompas, C. A., *Am. J. Physiol.*, in press.
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TABLE I. Data on Rats Under DCA, KCl, and DCA-KCl.

Treatments Group and No. rats	DCA-S* I (8)	DCA-KCl-S II (8)	KCl-S III (8)	None (4 on S) IV (8)
Heart wt: mg ± S.E.†	756 20	636 10	577 15	669 12
Dev. from prediction‡	+13%	— 4%	—14%	0%
Kidney wt: g ± S.E.	1.87 0.05	1.83 0.07	1.76 0.07	1.73 0.02
Dev. from prediction‡	+48%	+45%	+40%	+36%
Mesenteric periarteritis nodosa	5	0	0	0

* All animals were sensitized with 0.02% NaCl, excepting 4 of group IV.

† S.E. $= \sqrt{\frac{\Sigma (x-x')^2}{n(n-1)}}$. x' = mean value of x.

‡ Heart and kidney weights predicted from the data of Addis and Gray(8).

group I animals was due to ventricular hypertrophy (ascertained by histological study), was not due to increased water content (unpublished data), and was associated with mesenteric periarteritis nodosa in 5 of the 8 animals of the group. Periarteritis nodosa was not observed in any other area, or in any other group of animals in this study. Signs of glomerular zone hyperactivity of the adrenal, widening of the zone, hypertrophy of the cells, increased cholesterol content ("resistance phase")(?), associated with peripheral aggregation of ascorbic acid in many of the cells, occurred in the animals flooded with KCl. Glomerular zone atrophy, disappearance of its cholesterol, and subcapsular clumping of ascorbic acid, all signs of underactivity, were observed in the adrenals of Group I rats (DCA). The adrenals of the DCA-KCl (Group II) animals were of normal appearance except for disappearance of the transitional zone. Indeed this zone is very plastic even under normal conditions and its disappearance is probably of no significance.

Discussion. The data indicate that rats sensitized with NaCl and on DCA overdose develop cardiac hypertrophy and mesenteric periarteritis nodosa. This response was observed in previous instances(1,4,9) and was referred to the sodium retaining action of DCA. It has also been suggested that a normal balance exists between the amount of DCA-like corticoids in the body and the active renal mass(10); under this mechanism an excess of DCA supply to the animal disturbs this balance and elicits changes referable to the "non-inactivated" DCA, that is, cardiac hypertrophy, periarteritis nodosa and hypertension. A similar pattern of events may follow diminution of renal mass in which case even endogenous DCA-like corticoids may elicit the cardiovascular changes.

The observation that simultaneous KCl flooding prevented the signs of cardiac toxicity of DCA may be explained on the basis of either theory. The electrolyte could conceivably lead to sodium diuresis and in this manner depress the damaging effects of DCA. On the other hand, the excess KCl supply, through an increased need for the potassium eliminative function of corticoids, could have led to an increased "utilization" of DCA. With reference to the latter mechanism is the observation that the adrenal hyperactivity induced by KCl flooding is prevented by simultaneous DCA treatment. In other words, in KCl flooding, the adrenal responded by producing an increased amount of potassium chloride-depleting hormones, but this compensatory reaction did not occur in the presence of a high exogenous supply of DCA.

It is possible that the kidney participated in the changes observed in this study, but the exact nature of its participation is not known. Kidney hypertrophy was associated on the one hand with cardiac hypertrophy, periarteritis nodosa, and adrenal atrophy, and on the other hand with adrenal hypertrophy and a decrease of cardiac mass. Further studies are being conducted on the possible role of the kidney in these responses.

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Summary. The action of potassium chloride flooding on the cardiac, renal and adrenal effects of DCA-overdosage was investigated. The data indicate that there is a mutual antagonism between the actions of DCA and KCl on the heart and the adrenal. The role of the kidney in this antagonism is not known.

The author acknowledges the helpful interest shown by Dr. C. E. Leese. The DCA (Percorten, Ciba) was supplied through the generosity of Dr. F. E. Houghton, Ciba Pharmaceuticals, Inc., Summit, N. J.

Received January 29, 1951. P.S.E.B.M., 1951, v76.

Oral Administration of Vitamin B_{12} Containing Cobalt^{60} to Rats. (18501)

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Recent reports by Chow and associates (1,3) have demonstrated that when vit. B_{12} is administered orally to rats or humans, no microbiologic activity appears in the urine. They concluded from these observations that oral administration results either in the conjugation of vit. B_{12} to a form unavailable for the microorganism, in poor absorption in the intestinal tract, or in conversion to a form incapable of being excreted by the kidneys. Later they observed (unpublished work) that stool samples from rats receiving vit. B_{12} by mouth contained considerably more of this vitamin than stools excreted before administration. Although these data might be taken to indicate poor utilization of vit. B_{12}, this interpretation is complicated by the presence of some vit. B_{12} in the feces of untreated, normal rats and by the unreliability of the microbiologic assay for this vitamin in stool.

The availability of radioactive vit. B_{12} containing cobalt^{60} reported by Chaiet, Rosenblum and Woodbury(2) simplifies the analytical problem and permits a differentiation between the administered vitamin from that originally present, granting that the radioactivity due to cobalt^{60} found in the animal represents administered vit. B_{12}. It is therefore of interest to ascertain the distribution of vit. B_{12} as manifested by the presence of radioactive cobalt^{60} in urine and stool, as well as in certain organs of rats following oral administration. The results of such a study are reported below.

Experimental. Four normal rats weighing between 250 and 300 g were put into metabolism cages and fed a soy bean diet(3). Collections of urine and stool were made for 2 consecutive days. On the third day, 0.89 mg of radioactive B_{12},* with a specific activity of $\simeq 0.2$ microcurie per milligram, was administered orally to each rat. Daily collections of urine and fecal matter were again made for 3 to 4 consecutive days. On the fifth day after administration, the animals were sacrificed and the kidneys, liver, spleen, brain, testes and hearts removed. Each of these organs was homogenized *in toto* either with a Waring Blendor or with an Elvehjem homogenizer, and the suspensions were made up to a measured volume. An aliquot of each tissue homogenate was evaporated to dryness, wet ashed with H_2SO_4 after addition of several milligrams of ordinary cobalt to act

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2. Chaiet, L., Rosenblum, C., and Woodbury, D. T., *Science*, 1950, v111, 601.

3. Chow, B. F., Barrows, L., and Lang, C. A., *J. Nutrition*, in press.

* The radioactive vitamin employed in these experiments was provided by Merck & Co., Inc., on allocation by the Isotopes Division, U. S. Atomic Energy Commission.

as carrier, diluted to 10 ml in volumetric flasks, and assayed for cobalt[60] content. One ml samples of blood were wet ashed directly. The stools collected before administration of vit. B_{12} were combined in a single sample, and stools collected after administration combined into a second sample. Aliquots of each were assayed after wet oxidation. The daily samples of urine were assayed directly, after appropriate dilution, for both radioactivity and microbiological activity. Radioactivity measurements were performed with a windowless counter (Q-Gas Counter) using evaporation residues in shallow aluminum planchets 3 cm in diameter. Under these conditions, the specific activity of the radioactive vit. B_{12} was 221,000 counts per minute (cpm) per mg; hence 197,000 cpm was administered per rat. The radioactivity of all samples was measured first using residues obtained by direct evaporation of the acid digests after neutralization with ammonia. Self-absorption corrections, based on ammonium sulfate residues, were applied to all samples as required. In the case of blood, liver, testes, heart, spleen and brain digests, the activities determined by direct evaporation of aliquots were quite low and not distinctly different from the controls. Accordingly, larger volumes of these acid digests were neutralized and treated with ammonium thiocyanate to form the blue cobalt thiocyanate complex, which was then extracted with a mixture of ethyl ether and isoamyl alcohol in a 4 to 1 ratio(4). The entire organic extract was evaporated in aluminum planchets for radiometric observation. Planchets were heated on a hot plate to remove small amounts of thiocyanate transferred to the planchets by the organic solvent. Kidney digests from 3 of the rats were also treated by this extraction method, as was one of the feces samples. Satisfactory agreement between both methods was obtained in these cases. A reagent control was run simultaneously with the test experiments. Basal urine and stool collections served as controls for excrement radioactivity. Two rats were included as controls for the blood samples.

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TABLE I. Distribution of Radioactivity After Oral Administration of Vit. B_{12} Containing Cobalt[60].

	Avg* activity, cpm	Stand. dev., ± cpm	Vit. B_{12} equivalent, μg
Liver	940	760	4.3
Kidney	920	270	4.2
Blood (1 ml)†	13	7	$\sim<0.06$
Spleen	13	20	$\sim<0.1$
Brain	19	15	$\sim<0.1$
Testes	17	20	$\sim<0.1$
Heart	—8	14	—

* In all cases, measured control activities were ~3 cpm. After correction for sampling schedule, etc., the calculated control values employed were 40 ± 18 cpm for liver, 4 ± 6 cpm for blood, and 30 ± 13 cpm for remaining organs.

† Avg of 3 rats.

Results. Results of the distribution measurements are reported in Table I which gives the average total cpm for the 4 rats, (corrected for the control radioactivity), the standard deviation for these net activities, and the quantities (in micrograms) of vit. B_{12} corresponding to these net activities. In Table II is reported the elimination of B_{12} (in micrograms) in the stool and urine. Results of microbiological assays of urine specimens, determined by means of *L. leichmannii* activity(5), are also included.

In addition to observing the radioactivity, the 24-hour urine samples were examined further to ascertain whether the radioactivity was truly present as vit. B_{12} rather than as free cobalt. The vitamin is known to be extractable by water-immiscible alcohols from concentrated aqueous solutions of ammonium sulfate(6). Subjecting the urine samples to this extraction procedure resulted in the removal of 80-100% of all activity. Furthermore the distribution of the extracted radioactivity between benzyl alcohol and water was close to that expected(2,7) for vit. B_{12}. These tests rule out the presence of radioactive in-

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6. Fantes, K. H., Page, J. E., Parker, J. F. J., and Smith, E. L., *Proc. Royal Soc.*, 1949, v136-B, 592.

7. Anslow, W. K., Ball, S., Emery, W. B., Fantes, K. H., Smith, E. L., and Walker, A. D., *Chemistry and Industry*, 1950, No. 29 (7-22-50), 574.

TABLE II. Elimination (in μg) of Vit. B_{12} in Urine and Stool.

Sample	Time from admin.	Rat 1		Rat 2		Rat 3		Rat 4	
		Radioact.*	Microbial	Radioact.*	Microbial	Radioact.*	Microbial	Radioact.*	Microbial
Urine	−48			—					
	−24			—					
	+24	18.3	37.0	7.1	8.0	42.8	63.0	20.6	20.0
	+48	1.4	1.2	0.4	0.9	1.2	1.4	0.7	0.6
	+72	4.9	0.7	1.5	1.3	1.1	2.5		0.2
	+96	1.4	0.2	3.8	2.8				
	Total	26.0	39.1	12.8	13.0	45.1	66.9	21.3	20.8
Feces	−48 (combined)	0	—	1.4†	—	13.0†	—	0	—
	+72 (combined)	415	—	582	—	67.4	—	192	—

* Basal urine activities were negligible. Stand. dev. of ± 0.4-0.6 μg per measurement were estimated from counting statistics.

† These basal feces activities were traced to contaminated glassware used for wet ashing.

organic cobalt, although the possibility that other vit. B_{12}-like compounds(6-9) are partly responsible for the radioactivity of the urine cannot be excluded.

Discussion. It is immediately evident from these results that the amount of orally administered vit. B_{12} excreted in the urine of rats is indeed small compared to the large dose administered. The largest percentage urinary excretion observed in these experiments amounted to about 5% of the initial B_{12} in the case of rat 3 over a three-day period. It is further apparent that the most marked radioactive B_{12} elimination in the urine occurs within the first 24 hours, and beyond this initial period the amount tends to diminish to very low values. Results of microbial assay parallel the radioactivity findings although, due to the presence of vit. B_{12} or other active components of the rat diet, they may indicate larger amounts of B_{12}. One must conclude from the cobalt^{60} content of the urine that only a fraction of the excessive dose of vit. B_{12} administered in these experiments is eliminated through the urinary tract, and that earlier observations to this effect are not due to the existence of the vitamin in conjugated form unavailable to the test microorganism.

Concomitant with the low vit. B_{12} content of the urine is the high cobalt^{60} content of the feces. In the case of rat 2, the radioactivity was equivalent to $\simeq 65\%$ of the total radioactive B_{12} added to the diet. The fecal radioactivity of this and the other rats would undoubtedly have been even higher had the intestinal contents of the animals been removed for analysis at the time of autopsy. Whether this expedient would have accounted for the radioactive vitamin in its entirety is uncertain since a complete examination of the carcasses were not performed.

The dose of 0.89 mg of vit. B_{12} is, of course, far in excess of physiological requirements. Therefore, despite the low values found for B_{12} in the urine relative to the administered quantity, its very presence(4) proves that intestinal absorption occurs upon oral administration. This conclusion is supported by the presence of significant radioactivity, equivalent to $\simeq 0.5\%$ of radioactive B_{12} administered in the kidneys and livers. The radioactivity of the blood, though small, is an indication of the passage of B_{12} through the blood stream. The quantities listed (Table I) for the remaining organs are doubtful, and probably represent maximal amounts. Additional work with high specific activity vitamin(10) is planned to ascertain the extent of transfer of the vitamin to these latter organs.

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Received January 16, 1951. P.S.E.B.M., 1951, v76.

Mechanism of Protective Action of Glutathione Against Whole Body Irradiation.* (18502)

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The ability to modify the effects of *whole body* radiation was first proposed by Hektoen (1) who discovered an apparent protection by injection of foreign protein 10 days prior to radiation. Treadwell *et al.*(2) demonstrated protection by injection of estradiol 10 days before irradiation in mice. Ellinger(3) reported increased survival by post radiation treatment with desoxycorticosterone acetate. Patt *et al.*(4) and Cronkite *et al.*(5) have shown that pre-irradiation administration of sulfhydryl compounds will protect against radiation injury within certain defined limits. Dowdy *et al.*(6) have shown that anoxia during irradiation will afford significant protection. Miller(7) has shown that post radiation administration of antibiotics will increase the survival of mice exposed to a little less than an LD_{100}.

Upon conclusive proof that glutathione could protect against radiation injury(8) it was decided to make a systematic study of the comparative organ weights, complete blood counts, and histologic appearance of the tissues of the treated and untreated mice. These studies with technics will be reported in detail in a subsequent communication(9).

White Swiss inbred male mice weighing 20-24 g were irradiated with the radial beam of a 2.0 Mev. GE industrial X-ray tube. All

TABLE I. Splenic-thymic Weights in mg of Glutathione Treated and Untreated Male White Swiss Mic Exposed to 820 r (LD_{85}) Whole Body X-ray. Mean weight and range of 6 animals sacrificed on each day ar tabulated. Testicular weights are from the 750 r experiment.

		Days after irradiation							
		0	1	3	5	7	12	14	21
Thymus	Treated	19.8 14-28	15 12.0-19.2	7 5.4-9.6	7 4.0-11.8	8.1 3.4-13.2	27 7.0-39.8	35.7 5.2-120	14.6 4.8-35.4
	Untreated	19.8 14-28	16.8 11.8-22.6	8.6 6.8-10.0	6.2 4.0-10.0	7.2 2.4-12.0	10.1 4.0-15.8	6.6 3.2-12.8	8.6 2.6-12.4
Spleen	Treated	192 126-298	58.1 50.0-66.4	48.6 37.0-67.6	48.6 26.0-76.8	32.8 25.8-42.4	120.0 54.6-201	80.5 49.8-147	217.1 138.0-271.
	Untreated	192 126-298	66.8 55.2-79.0	47.9 30.8-94.0	44.5 19.0-67.8	48.9 23.2-66.4	48.0 38.0-57.4	86.4 44.2-197	206.5 87.8-210.
Testicle	Treated	182 151-202			159 139-199	145 119-189	109 82-149	111 84-148	94 57-123
	Untreated	182 151-202			163 117-204	142 120-156	132 106-151	109 87-132	83 71-97

* Opinions are those of the authors and are not to be construed as reflecting those of the U. S. Naval Service.

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TABLE II. Mean Blood Counts and Range of Glutathione Treated and Untreated Mice Sacrificed at Stated Intervals After Irradiation with 820 r. Mean and range is tabulated.

		Days after irradiation							
		0	3 hr	1	3	5	7	12	14	21
Leukocytes		7	9.2	3	1.2	.7	.7	.5	.5	5.5
× 10³/mm³	Treated	4.1-14.7	4.0-23.4	1.0- 5.9	.7- 1.8	.4- .9	.2- 1.6	.1- .9	.4- .6	2.0 -11.8
		7	4	2.4	1.3	.4	.2	.3	.9	5.3
	Untreated	4.1-14.7	2.3- 6.4	.9- 5.3	.5- .2	.2- .7	.1- .4	.1- .5	.4-2.7	1.8 -11.8
Granuloeytes		2.3	5.9	1.8	.5	.3	.3	.2	.3	3.8
× 10³/mm³	Treated	.9- 4.7	2.9-11.2	.4- 5.2	.3- .7	.2- .5	.06- .6	.01- .4	.1- .6	1.3 - 9.8
		2.3	2.3	1.8	.7	.03	.07	.06	.3	2.9
	Untreated	.9- 4.7	1.9- 3.7	.7- 4.4	.2- 1.2	—	.01- .1	.02- .16	.04-1.5	.99- 6.6
Lymphocytes		4.7	3.2	1.1	.4	.4	.3	.3	.2	1.6
× 10³/mm³	Treated	2.4-10	.4- 5.5	.5- 3.1	.3- .7	.2- .6	.04- 1.1	.09- .7	.1- .4	.7 - 3
		4.7	1.6	.7	.6	.3	.11	.2	.6	2.3
	Untreated	2.4-10	.6- 3	.2- 1	.3- .8	.01- .6	.09- .16	.08- .4	.3-1.2	.8 - 5.2
Red blood cells		9.8	10.7	8.8	9.9	8.6	8.2	5.9	5.5	7.7
× 10⁶/mm³	Treated	7.9-11	8.8-12.2	7.7- 9.3	8.0-11.5	7.8-10.2	7.4-10.2	4.2 -7.6	2.8-7.5	5.7- 9.1
		9.8	10.3	9.6	9.4	8.6	8.0	5.2	4.6	6.1
	Untreated	7.9-11	9.0-12.2	8.4-12.1	8.1-10.6	7.5-10.1	5.7- 9.1	2.6-7.1	2.4-7.4	2.0 - 7.9

mice were given 820 r with the exception of the mice in which the testicles were studied. The latter received 750 r. Before irradiation the mice received either 4 mg of glutathione per gram of mouse subcutaneously as a 10% solution at pH 6.5 or isotonic saline in the same volume.

In Table I are shown the comparative changes in the weights of radiosensitive organs of the treated and the untreated mice after exposure to 820 r whole body X-ray.

In Table II are seen the mean blood counts of treated and untreated mice. Sections of bone marrow, thymus, spleen and lymph nodes showed no definite differences in the rate and extent of destruction of hemopoietic tissues of treated and untreated animals killed 1 and 3 days after irradiation. The subsequent regeneration of myelopoiesis in the bone marrow and in the pulp of the spleen was markedly accelerated in the treated animals. Differences in the regeneration of the lymphoid tissues were only slight, but appeared compatible with the concept of more rapid regeneration in the treated animals which was suggested by the greater organ weight gains in the treated animals.

Discussion. The similarity of the glutathione treated and untreated irradiated mice in respect to their radiosensitive organ weights, blood counts and histologic picture during the first 3-5 days is highly suggestive that cellular destruction of radiosensitive tissues (bone marrow, thymus, spleen, and testis) is of equal degree in the two groups. It is impossible to prove this conclusively by histologic technics because a few blasts might survive and be undetected.

In contrast to the similarity in the destructive phase the bone marrow, spleen, and thymus regenerated much more rapidly in the glutathione treated animals as judged by the criteria used.

The leukocytes in the peripheral blood show very little difference between the treated and the untreated mice, but this is not surprising because it has been shown(10) that the levels of leukocytes in peripheral blood do not

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necessarily reflect the histologic activity of the hemopoietic tissues. The decrease in the red blood counts during the first 7 days is similar. The Rbc's of the glutathione treated mice do not fall as low thereafter and recover more rapidly.

The testicular injury by histologic criteria and testicular weight is identical in both groups over a 21 day period during which there is no evidence of regeneration in either.

It appears that glutathione by an unknown manner protects the mechanisms which accelerate the regeneration of the hemopoietic tissues but does not prevent the cellular destruction. This concept is not proved. Evidence collected elsewhere lends support. Trowall(11) has shown that cysteine does not protect lymph node cultures. Hennessey (12) by a precise technic has shown that neither glutathione nor cysteine protect against the radiation inhibition of iron uptake by the erythropoietic tissue.

Conclusions. 1. By the criteria used there is no significant difference in the destructive effects of irradiation on normal mice or mice treated with large doses of glutathione before irradiation. 2. A striking difference is seen in the rate of regeneration of the hemopoietic tissues.

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Received January 24, 1951. P.S.E.B.M., 1951, v76.

Acid-Binding Capacity of Dialyzed Mucin Fractions from Human Gastric Juice.* (18503)

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(Introduced by D. P. Barr.)

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The mechanisms responsible for variations in gastric acidity are still incompletely understood. Hollander(1) has shown that hydrochloric acid is secreted in constant concentration at 0.17 normal, but at a variable rate. It has generally been assumed that variations in gastric acidity are attributable not only to dilution but to the partial neutralization of the hydrochloric acid by the constituents of the gastric mucus, although full data on the buffering power of human gastric mucin are not available. By the method of Glass and Boyd(2) the chief constituents of the gastric mucin can be determined separately. These authors found that a mucous substance which they called mucoprotein is secreted by the glandular cells, and that another constituent of the dissolved mucin, which they called mucoproteose, is derived from the visible mucus fraction which in turn is secreted by the lining columnar epithelium. Which of the mucous substances or their degradation products is chiefly concerned with neutralizing hydrochloric acid is still not known and there is little agreement to be found in the literature concerning the buffering role of gastric mucus. Some workers have attributed a marked buffering effect to the mucous substances(3), others, a moderate effect(4), and

* We are indebted to Dr. Vincent du Vigneaud and Dr. Julian Rachele of the Biochemistry Department, Cornell University Medical College, for their generous assistance.

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others, a negligible effect(5). The discrepancies in results seem most probably to be related to differences in methods of preparing the mucous substrate for testing, and perhaps also to the fact that these substances were derived variously from animals and humans and some may have contained contaminating substances from the saliva and regurgitated bile.

The present investigation concerns an inquiry into the buffering capacity of mucous substances extracted and refined from the stomach of Tom, a fistulous human subject with a completely occluded esophagus which precludes contamination of the gastric juice with salivary and nasopharyngeal mucus(6).

It has been shown earlier that the gastric juice of Tom is poor in mucoprotein. Therefore, no attempt was made to extract sufficient mucoprotein for the testing procedure from his gastric juice. Instead, pooled gastric juice rich in mucoprotein was collected 40 to 60 minutes after insulin stimulation from five patients with active duodenal ulcer(7).

Material and methods. The material tested was prepared as follows:

A. *Visible mucus from the surface epithelium.* The gastric content of Tom was collected under fasting conditions directly from his fistulous opening. The visible mucus fraction was separated by centrifugation from the supernatant fluid of the gastric juice, pooled and refrigerated under 0.05 normal hydrochloric acid. Fifteen cc of this material were placed in a Visking bag and dialyzed against flowing tap-water at 6°C for 48 hours. The material was then homogenized by shaking and again dialyzed in a parchment dialyzing shell against distilled water for another 48 hours at 6°C. The material was not considered suitable for use until its specific conductance was smaller than 0.0001 reciprocal ohms. Five samples of Tom's visible gastric mucus were prepared in this fashion. The content of mucoid substance (dry weight) in tested samples ranged from 16.0 to 50.0 mg.

B. *Dissolved mucoproteose.* After separation of the visible mucus fraction from the pooled gastric juice of Tom, the remaining supernatant fluid was mixed with one-half volume of 10% trichloracetic acid. After it was allowed to stand for 15 minutes, the mixture was centrifuged and the supernatant fluid was decanted from the precipitate. One and one-half volumes of acetone were then added to the supernatant fluid. This mixture was left for one hour in an incubator at 40°C. The resulting precipitate was then collected by centrifugation, was washed with acetone-water and then pure acetone, and was finally redissolved in 0.1 normal sodium hydroxide. To this solution was added 1½ volumes of 0.1 normal hydrochloric acid and 2½ volumes of distilled water. This mixture was allowed to stand until a precipitate settled out and then centrifuged. The supernatant fluid was separated, mixed with two volumes of acetone and left for 24 hours at 37°C. The resulting precipitate from this operation was collected, washed with acetone-water and dialyzed as under "A." The final material recovered was then divided into two parts, each of them containing about 50 mg of dry weight substance. Each was titrated as below and subjected to analysis for mucoproteose by the method of Glass and Boyd (2).

C. *Dissolved mucoprotein.* The separation of mucoprotein from the pooled gastric juice of 5 patients with duodenal ulcer was carried out as described under "B" until the stage of precipitation by hydrochloric acid. The precipitate thus obtained was washed with acetone-water and pure acetone and then dialyzed as under "A." The recovered material was then divided into two parts, each of them containing about 50 mg of dry weight substance. Each was titrated as outlined be-

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low and each was analyzed for the presence of mucoprotein by the method of Glass and Boyd(2).

Testing procedure. The dialyzed material was suspended and homogenized in 5 to 6 cc of redistilled water before titration. Each of the fractions was then titrated electrometrically with 0.1 normal and 0.5 normal hydrochloric acid. The titration was accomplished using the Beckman Research Model Potentiometer with glass electrode and the Koch Automatic Microburette, calibrated in 0.01 cc and equipped with a ground glass tip for delivery of the smallest possible drop. Control titration curves were obtained using redistilled water in volume equal to the dissolved mucous substances. All titrations covered a pH range from approximately 6.0 to 1.6. Following titration, the material was carefully collected and re-analyzed for its content of visible mucus, mucoproteose or dissolved mucoprotein respectively. After construction of the titration curves, the control curves obtained with the distilled water were deducted, thus establishing the buffering capacity of each tested material per gram of dry mucous substance at pH 2, 2.5 and 3. The acid binding capacity below this range

TABLE I. Results of Electrometric Titration of Dialyzed Samples of Surface Epithelium Mucus, Dissolved Mucoproteose, and Dissolved Mucoprotein from the Gastric Juice of Man.

Material tested	Acid binding capacity of 1 g of mucous substance tested in cc 1/10 N HCl		
	pH 3.0	pH 2.5	pH 2.0
A. Dialyzed pooled gastric surface epithelium mucus from Tom			
Sample #1	2.51	3.14	4.70
" 2	5.40	6.24	7.55
" 3	5.00	5.80	6.60
" 4	3.12	3.69	4.40
" 5			5.77
Avg with stand. error	4.01 ± .71	4.72 ± .76	5.80 ± .39
B. Dialyzed pooled gastric dissolved mucoproteose from Tom			
Sample #1	3.25	4.10	5.28
" 2	3.75	4.73	5.92
Avg	3.51	4.42	5.60
C. Dialyzed pooled gastric dissolved mucoprotein from 5 individuals			
Sample #1	3.18	3.73	4.32
" 2		3.56	4.27
Avg	3.18	3.66	4.31

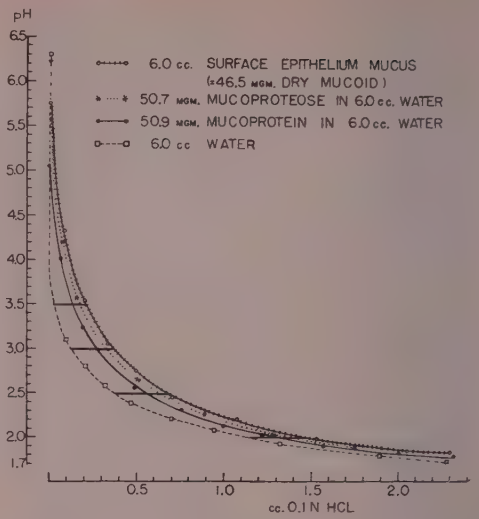

FIG. 1.

Typical electrometric titration curves of surface epithelium mucus, mucoproteose, and mucoprotein from human gastric juice. Horizontal lines connecting the curves with the curve of the H_2O control indicate the acid-binding capacity of the mucous substances at a given pH in cc of 0.1 N HCl.

was not used for calculations because of possible error in the potentiometer at a low pH and errors due to the dilution.

Results. Visible mucus. The data obtained on the 5 pooled samples of visible gastric mucus from Tom's stomach are reported in Table I, and one of the typical titration curves is shown in Fig. 1. The average acid binding capacity of the dialyzed gastric visible mucus calculated with standard error was found to be 0.41 ± 0.07 mEq at pH 3, and 0.58 ± 0.06 mEq at pH 2 calculated per gram of dry substance of visible mucus, and 0.31 ± 0.05 mEq at pH 3, and 0.44 ± 0.045 mEq at pH 2 per 100 cc of native mucus.

In order to obtain an estimate of what part the visible gastric mucus might play in buffering gastric acid, the fasting content of Tom's stomach was collected for 20 consecutive days. The total volume, and free and total acidity were measured, and the content of visible mucus was determined volumetrically under standard conditions, that is, after centrifugation for 10 minutes at 3,000 r.p.m., and these quantities were multiplied by the values obtained above. It was found, as indicated in

TABLE II. Data on Fasting Gastric Secretion Recovered from Tom's Stomach.

Fasting gastric specimens from Tom	Avg value representing means with stand. error from 20 tests performed on as many different days
Volume in cc	19.4 ± 4.0
Conc. of HCl in mEq. per 1*	41.0 ± 2.4
Content of HCl in mEq.	.79 ± .17
Volume of visible mucus in cc	1.68 ± .05
Content of mucoid of visible mucus (dry wt) in mg†	12.7 ± .4
Acid binding capacity of visible mucus in collected‡ specimen at pH 2 in mEq.	.0074 ± .0002
% of HCl contained which may be buffered at pH 2 by visible mucus present in collected specimen	.93 ± .20

* Only acid containing specimens were considered.

† The dry wt of the mucoid substance calculated on basis of values reported previously(2) and which avg 0.757 g per 100 cc native mucus.

‡ Acid binding capacity of mucus calculated on basis of avg value reported in this paper, *i.e.*, 0.58 mEq. per 1 g dry mucoid substance.

Table II, that the visible gastric mucus recovered from the fasting gastric juice of Tom may not bind more than 1% of the free acid present in Tom's stomach under average fasting conditions.

Comment. It is clear from these experiments that the buffering capacity of dialyzed visible mucus is much less than that of undialyzed material from humans or animals (3,4). It seems likely that the buffering capacity of the undialyzed material depends chiefly on the presence in the mixture of bicarbonate radicals bound to mineral bases (5) and the dialyzable products of protein breakdown.

Dissolved gastric mucin. In Table I and Fig. 1 are represented the acid binding capacities of the 2 main components of the dissolved gastric mucin, mucoproteose and mucoprotein. The acid binding capacity of the dialyzed mucoproteose from Tom's stomach was found to be approximately 0.35 mEq at pH 3 and 0.56 mEq at pH 2, and that of mucoprotein 0.32 mEq at pH 3, and 0.43 mEq at pH 2, calculated per gram of dry substance. For purposes of comparison it may be noted that the acid binding capacity of commercially available crystallized pepsin (Armour) was found to be 0.42 mEq at pH 3 and 0.61 mEq at pH 2 per gram of dry substance.

In an attempt to calculate the role of these purified substances in the buffering of the gastric juice, the calculations listed in Table III were made. As a basis for these calculations, quantitative data on the content of the various secretory constituents in the gastric juice were used, which have been reported separately elsewhere(8).

These calculations indicate that the mucous substances dissolved in the gastric juice may participate to some extent in the buffering of the gastric acid exclusive of their content of electrolytes, amino acids, peptides, and other dialyzable substances. In each instance, the magnitude of the final buffering effect would depend mainly upon the rate of acid secretion prevailing at the time. In individuals who may secrete very little acid, approximately 10% of the acid under fasting conditions is capable of neutralization by the dialyzed mucous substances. In more active stomachs, however, despite the greater output of mucous substances, the percentage of acid which the dialyzed mucous fractions can neutralize under fasting conditions is much lower, and about only one-half of the above value. Following stimulation of hydrochloric acid secretion by insulin, still less acid can be neutralized (3.9 ± 1.4%), although secretion of the total mucous substances may be greatly enhanced. After histamine stimulation which does not cause a significant increase in secretion of gastric mucin a still smaller percentage of total gastric acid secreted can be buffered by mucin fractions dissolved in the gastric juice (not more than 1.2 ± 0.4%).

Summary and conclusions. Electrometric titration of dialyzed visible gastric mucus recovered directly from the stomach of a man with a large gastric fistula and an occluded esophagus revealed an acid binding capacity, if calculated per gram of the dry substance, far smaller than that reported for

8. Glass, G. B. Jerzy, and Boyd, L. J., *Gastroenterology,* 1950, v16, 697.

TABLE III. Data on Gastric Secretion Collected Under Fasting Conditions and After Stimulation with Insulin and Histamine and Calculated on the Basis of Secretory Patterns Reported Elsewhere(8). All values are calculated as means with standard errors, and are based on number of tests indicated below.

	Fasting gastric juice collected twice at intervals of 20 min.		Gastric juice collected within 1 hr after i.v. inj. of 16 U insulin		Gastric juice collected within 1 hr after inj. of 1 mg histamine	
Groups of individuals tested	Controls	Duodenal ulcer	Controls	Duodenal ulcer	Controls	Duodenal ulcer
No. of individuals tested	35	27	9	18	12	7
Vol. in cc	20 ± 3	34 ± 5	60 ± 10	128 ± 12	78 ± 10	123 ± 16
HCl						
Conc. in mEq./l	15 ± 3	30 ± 4	34 ± 6	64 ± 5	60 ± 8	89 ± 8
Content in mEq.	.3 ± .1	1 ± .2	2.2 ± .7	8.2 ± 1.5	4.7 ± 1.3	10.9 ± 2.1
Dissolved mucoprotein						
Conc. in mg/100 cc	48 ± 8	66 ± 9	90 ± 22	136 ± 12		
Content in mg	12 ± 2	23 ± 4	59 ± 17	174 ± 33		
Acid-binding capacity in mEq.*	.005 ± .001	.010 ± .002	.025 ± .007	.074 ± .014		
Dissolved mucoproteose						
Conc. in mg/100 cc	237 ± 26	156 ± 28	233 ± 35	144 ± 18		
Content in mg	46 ± 6	65 ± 19	107 ± 30	183 ± 35		
Acid-binding capacity in mEq.*	.026 ± .003	.036 ± .011	.060 ± .016	.102 ± .019		
Total dissolved mucin						
Conc. in mg/100 cc	285 ± 27	222 ± 29	323 ± 41.3	280 ± 21.6	136 ± 28	151 ± 17
Content in mg	58 ± 6.3	88 ± 19.4	166 ± 34.5	357 ± 47.9	106 ± 11	184 ± 22
Acid-binding capacity in mEq.*	.031 ± .003	.046 ± .012	.085 ± .017	.176 ± .024	.053 ± .006	.092 ± .011
% of HCl contained in collected gastric juice which may be buffered by total dissolved mucin at pH 2	10.3 ± 3.5	4.6 ± 1.3	3.9 ± 1.4	2.1 ± .5	1.2 ± .4	.8 ± .2

* The acid-binding capacities were calculated on the basis of avg buffering values of the dissolved mucoprotein and mucoproteose reported in this paper.

undialyzed human or animal mucus; the acid binding capacity for mucoproteose and mucoprotein dissolved in gastric juice was even smaller than that for visible mucus. This suggests that dialyzable materials including mineral bases are chiefly responsible for the alkalinity of gastric mucus and the buffering of the HCl of the stomach. The total buffering capacity of mucous substances dissolved in gastric juice ranges from 10.3 ± 3.5 to 1.2 ± 0.4% of the free acid present, depending upon the rate of acid and mucin secretion prevailing at the time. It is emphasized that this study has concerned dialyzed and refined mucous substances in the gastric juice. These experiments do not bear upon the ability of the visible gastric mucus which invests the lining of the mucous membrane of the stomach to protect the underlying columnar epithelial cells by physical means (9).

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Received December 15, 1950. P.S.E.B.M., 1951, v76.

Some Actions of Mercury Compounds on the Heart.* (18504)

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Severe impairment in cardiac function can be produced by the intravenous injection of mercurial diuretics(1-3). The object of the present investigation was to relate the dosage of the mercurials to the changes in the electrocardiogram and to determine differences in cardiac action of various types of mercury compounds.

Methods. All experiments were performed on mongrel dogs weighing 6 to 13 kg. The anesthetic used was Dial Urethane§ (0.6-0.7 cc per kg given intraperitoneally). The average arterial pressure was recorded from the common carotid or the femoral artery by means of a mercury manometer. Electrocardiographic tracings were obtained by means of subcutaneous leads, lead 2 being usually employed. Records were taken on an ink-writing oscillograph. In 3 experiments leads were taken directly from the auricle or ventricle by means of small metal clips attached to the respective organs. The mercurial to be studied was infused at a rate of 3 micromoles per kg per minute into the femoral vein by means of a constant rate infusion pump. The mercury compounds employed were mersalyl‖ (2-[(2-hydroxy-mercuri-3-methoxypropyl) carbamyl] phenoxy-acetic acid) mersalyl with theophylline,‖ Esidron acid§ (2-[2-hydroxy-mercuri-3-hydroxy-propyl-carbamyl] nicotinic acid) Esidron acid with theophylline,§ p-chloromercuribenzoate and mercury bichloride. Sodium salts of these compounds, except mercury bichloride, were prepared by the addition of equivalent quantities of sodium hydroxide.

Results. Following the infusion of a mercurial diuretic such as mersalyl or Esidron acid with and without theophylline, a fairly constant sequence of events occurred in the electrocardiograms taken either by conventional or by direct leads from the heart. The earliest changes observed were a depression and change in configuration of the ST segment, the "T wave" originating from the S wave. Changes in the height of the T wave were determined by the original state of this wave during the control period. If the T wave was upright, there were usually the ST changes and an increase in the height of the T wave; if inverted the main changes were the ST changes described above. The distance from S to the end of the T wave was

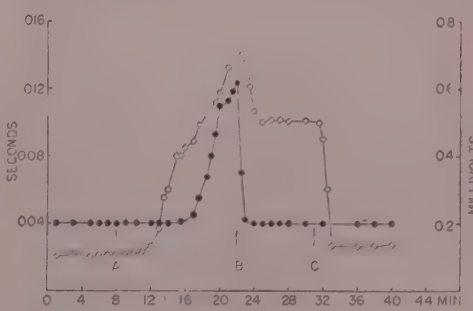

Fig. 1.

Effect of cysteine on electrocardiographic changes produced by free Esidron acid. Male dog 8.6 kg ECG Lead II. At A: infusion of Esidron (3 micromoles per kg per min.) was started. At B and C: 2 and 5 mg per kg cysteine hydrochloride respectively given by vein. Abscissa, time in min. Ordinates, the downward displacement of the ST segment in millivolts, or length of the QRS in sec. ●——● QRS in sec. ○——○ Displacement of the T wave in millivolts.

* Supported by a grant in aid from Ciba Pharmaceutical Products, Inc., Summit, N. J.

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2. Long, W., and Farah, A., *J. Pharmacol. Exp. Therap.*, 1946, v88, 388.

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§ Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

‖ Kindly supplied by Winthrop-Stearns Research Institute, Rensselaer, N. Y.

TABLE I. Effect of Mercury Compounds on the ECG of the Anesthetized Dog. Constant infusion of the mercurial at a rate of 3 micromoles per kg per min. All values are in micromoles.

Compound	Min. T changes	Min. QRS changes	Toxic dose*	Lethal dose†	No. of exp.
Free salyrgan	16.8 ± 6.1	28.9 ± 3.2	44.2 ± 4	48.6 ± 5	6
Free Esidron	10.2 ± 3	16.9 ± 5.2	28.7 ± 32	33.5 ± 2.9	7
Esidron with theophylline	12.8 ± 2.7	18.8 ± 3.6	32.7 ± 2.8	37.9 ± 3.4	6
Mercury bichloride	11.8 ± 4.5	16.0 ± 5.0	28.0 ± 7.2	31.0 ± 6.1	3
P chloromercuribenzoate	25.2 ± 8	—	—	160 ± 20	5

* Dose which doubles the width of the QRS complex.
† Dose producing ventricular fibrillation or cardiac arrest.

not changed appreciably even during the severe stages of poisoning with the mercurial diuretics. Changes in the QRS wave occurred somewhat later than the T wave changes and were characterized by widening and notching of the QRS complex (Fig. 1). With lethal doses of the above mercurial diuretics there was a period of ventricular tachycardia followed by ventricular fibrillation. In Table I are given the dosages of some mercury compounds producing the various electrocardiographic changes described above.

It can be seen from Table I that with all compounds the T wave changes were the first to appear. All compounds except p-chloromercuribenzoate produced widening of the QRS wave. Parachloromercuribenzoate is unique in this series since in the doses employed it produced only T wave changes with no change in the width of the QRS even following lethal doses.

The other changes observed with the mercurial diuretics containing no aminophylline are widening of the P wave and prolongation of the PR interval. The prolongation of the PR interval may occur before or after the beginning of the QRS changes. With the aminophylline containing diuretics Esidron and mersalyl the PR changes are minimal or absent. All the above changes are readily reversed by an adequate dose of a cysteine HCl or 2,3-dimercuptopropanol (BAL) (2). The observation has been made that following the injection of a thiol the ST and T wave changes produced by the mercurial diuretics are slower to respond than the QRS complex. With small doses of cysteine it is possible to correct the QRS changes while the T wave is only partially corrected. By increasing the dose of the sulfhydryl compound the T wave changes also disappeared (Fig. 1).

Heart rate and PR interval changes produced by the various mercurial diuretics are not the same. With the aminophylline free mercurial diuretics mersalyl and Esidron acid, an increase in heart rate occurred only during the very late stages of poisoning and was due to ventricular tachycardia. The PR interval was increased with both the free compounds and both ventricular tachycardia and PR prolongation were readily reversed by the intravenous injection of cysteine (Fig. 2A). With the theophylline-containing mercurial preparations Esidron and mersalyl there was a 20 to 30% increase in sinus heart rate which started during the early stages of the injection period. The PR changes were minimal and a slight increase occurred only during the latter stages of the poisoning. The tachycardia could not be abolished by cysteine, thioglycolic acid or BAL. The PR interval was reduced below control levels by these thiols (Fig. 2B). Since the above differences could be due to the theophylline content of these compounds, aminophylline was infused in 2 dogs at a rate of 3 micromoles per kg per minute. There was first a cardiac slowing lasting 30 to 60 seconds followed by an increase in heart rate and some decrease in the PR interval. Neither heart rate increase nor PR change could be affected either by BAL or cysteine (5 and 10 mg per kg respectively).

The action of p-chloromercuribenzoate. The intravenous infusion of this compound at a rate of 3 micromoles per kg per minute resulted in marked ST and T wave changes. No QRS changes were observed even with doses 2 to 3 times as great as the lethal dose of the other mercurials studied (Table I

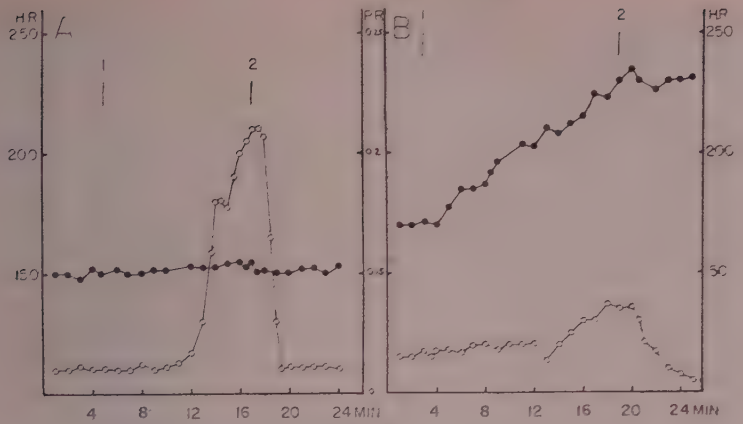

FIG. 2.

Effect of free Esidron and Esidron with theophylline on heart rate and PR interval in the anesthetized dog. A. Female dog 6.6 kg. 1: Start of the infusion of free Esidron (3 micromoles per kg per min.). 2: Intravenous inj. of 10 mg per kg of thioglycolic acid. B. Male dog 9.4 kg. 1: Start of infusion of Esidron with theophylline (3 micromoles per kg per min.). 2: Intravenous inj. of 10 mg per kg of thioglycolic acid. ○ —○ PR interval in sec. ● —● Heart rate beats per min. Abscissa, time in min. Ordinate heart rate beats per min. or PR interval in sec.

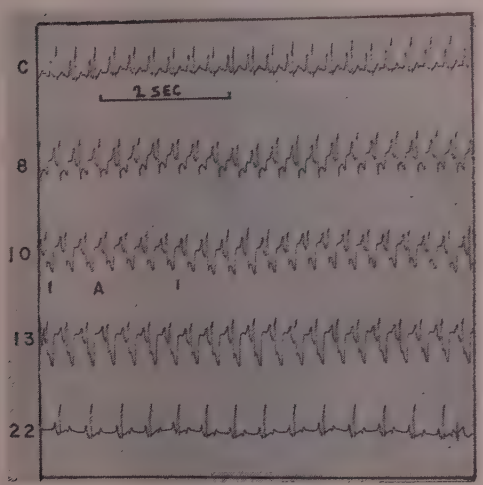

FIG. 3.

Effect of p-chloromercuribenzoate on the ECG of the dog. C. Control tracing. Between first and second tracing p-chloromercuribenzoate infused intrav. at 3 micromoles per kg per min. Numbers on left indicate time in min. following the start of infusion of p-chloromercuribenzoate. At A 20 mg per kg of cysteine hydrochloride was given intrav. At 21 min. 5 mg per kg of BAL was given intrav. Last tracing is after BAL inj.

and Fig. 3). With the mercurial diuretics and mercury bichloride death was due to ventricular fibrillation while with p-chloromercuribenzoate death was due to ventricular asystole. Prior to this ventricular asystole, there was a reduction in heart rate, a prolongation of the PR interval, followed by complete block, and appearance of slow ventricular extrasystoles. The slowing in heart rate and the prolonged PR interval could be corrected by the intravenous injection of cysteine hydrochloride (10-20 mg/kg) or BAL (5 mg per kg). Cysteine, even in large doses (20-40 mg/kg) had little effect on the T wave changes produced by toxic doses of p-chloromercuribenzoate while 5 mg per kg of BAL readily corrected these T wave changes (Fig. 3). The acute lethal dose of p-chloromercuribenzoate is about 160 micromoles and is high when compared with those of the mercurial diuretics and mercury bichloride (Table I). The probable reasons for this are the different mechanisms for the production of cardiac death described above.

Mercury bichloride has a cardiac action similar to that of the free mercurial diuretics and it produced the characteristic ST, T, QRS and PR changes. The administration of 10 to 20 mg per kg of cysteine corrected these changes only for 10 to 20 minutes, while with 10 mg per kg of BAL cardiac pro-

tection lasted several hours (3 experiments).

Discussion and summary. The various mercurials studied show both quantitative and qualitative differences in their cardiac action. In the case of the mercurial diuretics the aminophylline containing compounds produce an increase in the sinus rate as well as ventricular tachycardia while the free mercurials produce only a ventricular tachycardia (Fig. 2). The evidence presented makes it probable that the differences in response of the heart rate are due to the theophylline content of these preparations.

The ST and T wave changes described occurred somewhat prior to the QRS changes. ST and T wave changes are probably related to alterations in the process of repolarization of cardiac muscle while the QRS changes probably indicate mainly changes in intraventricular conduction. The mercurials studied are powerful inhibitors of enzymes containing essential SH groups(4,5). Thus the biochemical processes responsible for repolarization of cardiac muscle seem to be more sensitive to these SH inhibitors than the processes involved in intraventricular conduction.

The action of p-chloromercuribenzoate is of interest. It affects only the ST and T wave and thus probably interferes mainly with repolarization of the muscle but has no effect on intraventricular conduction processes. However, it does interfere with atrioventricular conduction and thus, with this enzyme poison it seems possible to inhibit selectively atrioventricular conduction. Differences in the histology and staining qualities of the atrioventricular and intraventricular conductile system have been described by Robb *et al.*(6) and pharmacological differences are thus not surprising.

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Received January 23, 1951. P.S.E.B.M., 1951, v76.

Prolongation and Potentiation of Anabolic and Androgenic Effects of Steroids: Testosterone and Methylandrostenediol.*† (18505)

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Carlinfanti *et al.*(1,2) have reported the potentiation and prolongation of the androgenic activity of testosterone in castrated male guinea pigs by the addition of aluminum phosphate to an aqueous suspension of the steroid. Margolin *et al.*(3) produced similar results in the albino rat. Both groups of workers have limited their reports to the androgenic effects. By use of a recently described assay procedure for anabolic activity (4), methylandrostenediol (MASD) has been found to be an anabolic agent which, in ordinary doses, has little androgenic activity (5). It was the purpose of our investigation to compare the duration and degree of action of the androgenic and anabolic properties of aqueous suspensions of MASD and of testo-

* Aided by grants from the Upjohn Co., Kalamazoo, Mich., and Ayerst, McKenna, and Harrison, Ltd., Rouses Point, N. Y.

† The methylandrostenediol was generously donated by Dr. K. W. Thompson, Organon, Inc., Orange, N. J.; testosterone cyclopentylpropionate by Dr. H. F. Hailman, The Upjohn Co., Kalamazoo, Mich.

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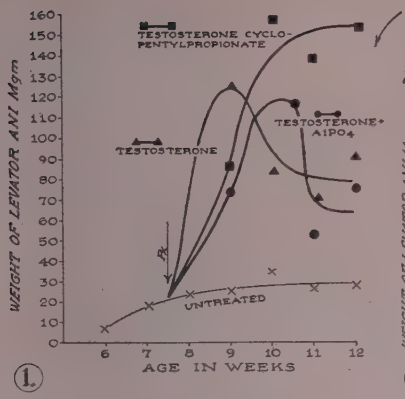
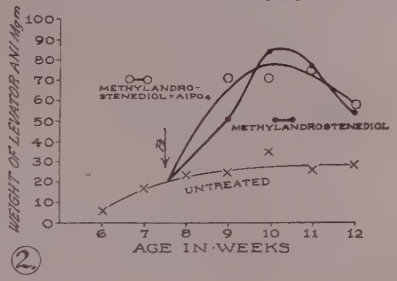
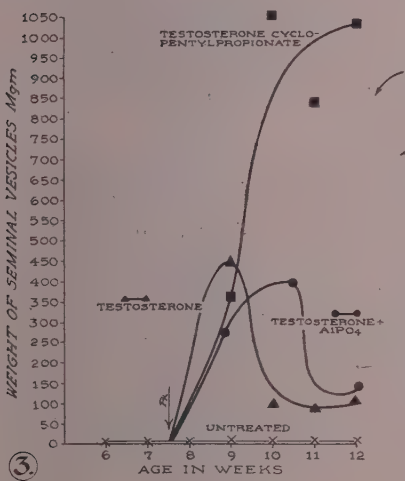


1. Effect of Testosterone, Testosterone + $AlPO_4$, and Testosterone Cyclopentylpropionate upon Levator Ani of castrated male rats.

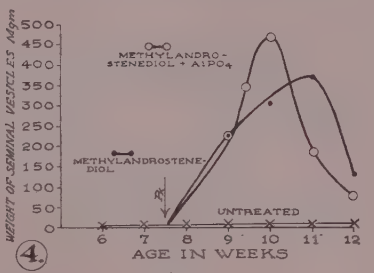
2. Effect of Methylandrostenediol and of Methylandrostenediol + $AlPO_4$ upon Levator Ani of castrated male rats.





3. Effect of Testosterone, Testosterone + $AlPO_4$, and Testosterone Cyclopentylpropionate upon seminal vesicles of castrated male rats.

4. Effect of Methylandrostenediol and of Methylandrostenediol + $AlPO_4$ upon Seminal Vesicles of castrated male rats



sterone, with and without added aluminum phosphate 3 mg and aluminum stearate 5 mg per 25 mg of steroid. Testosterone cyclopentylpropionate in sesame oil (TCP) was also studied because of the reported depot effect of this esterified form of testosterone (6), and for comparison with the aluminum phosphate suspensions.

Method. Male rats of the Long-Evans strain were castrated at the age of 4½ weeks. At the age of 7½ weeks, each was injected subcutaneously with a single dose of 25 mg of the agent. Groups, consisting usually of 5 animals but occasionally of 7, were autopsied at the ages of 9, 10, 11, and 12 weeks. Control groups of castrate untreated rats were autopsied at 6, 7, 8, 9, 10, 11, and 12 weeks of age to observe the sequence of events following castration. Androgenic activity was assayed by the weight of the seminal vesicles, and anabolic potency was estimated by the weight of the levator ani muscle, using the technique described elsewhere(4).

Experimental data and discussion. The results of this experiment are presented in Fig. 1-4.

It will be noted that in the absence of gonadal steroids (castrate untreated animals), the weight of the seminal vesicles remains plateaued, indicating that androgenic activity is lost. In contrast, the levator ani muscle continues to grow in the growing castrate ani-

6. Ott, A. C., Kuizenga, M. H., Lyster, S. C., and Johnson, B. A., personal communication.

mal but more slowly than in the intact animal(4).

Comparing the anabolic effects of the various agents in the dose employed in this study, it will be noted that esterification of testosterone with cyclopentylpropionic acid (Fig. 1) increases and prolongs anabolic activity to the greatest degree. With this agent (TCP) the anabolic effect is still maximal a month after injection. Aluminum phosphate delays both the measured actions of testosterone (Fig. 1 and 3) but does not significantly potentiate or prolong either effect of this agent. In the case of MASD (Fig. 2) there is no significant difference between the curves obtained with and without aluminum phosphate, probably because the steroid is highly insoluble. All the agents employed in this assay produce a great increase in the size of the levator ani muscle.

Androgenic activity is manifested by all these agents in the very large dose used. Aluminum phosphate does not delay, potentiate, or prolong the androgenic action of MASD (Fig. 4), presumably because of the solubility properties of the agent itself. Aluminum phosphate delays and somewhat prolongs the androgenic action of testosterone (Fig. 3); however, no great potentiation, *i.e.* increased efficacy, of this suspension is observed. Testosterone cyclopentylpropionate is the most potent agent in this group (Fig. 3). Its increased activity cannot be explained on the basis of slower release of the active constituent from the injection site alone, since there is no greater delay in growth of the levator ani muscle and seminal vesicles with the cyclopentylpropionic acid ester than with the aluminum phosphate suspension of testosterone. Inasmuch as the increased efficacy of this ester cannot be accounted for on the basis of delayed absorption, it seems likely that the long side chain delays the inactivation of the compound. This conclusion is in harmony with that of Miescher *et al.*(7) that the longer the carbon side chain, the more protracted is the effect. However, TCP differs from the esters studied by Miescher *et al.* in that prolongation of effect is not combined with decreased intensity of effect.

Caution must be exercised in transferring these observations to other species since, as Miescher *et al.* have pointed out, rats are more sensitive to the action of testosterone esters than are capons.

Summary. 1. Testosterone cyclopentylpropionate shows the greatest anabolic and androgenic efficacy of the agents studied. Its increased efficacy cannot be explained on the basis of delayed absorption, suggesting a delayed inactivation of this ester. 2. Aluminum phosphate delays the onset and decline of the anabolic and androgenic actions of testosterone in aqueous suspensions, but produces no such effect upon the actions of methylandrostenediol.

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Received January 17, 1951. P.S.E.B.M., 1951, v76.

Purification and Identification of the Antistiffness Factor.* (18506)

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In a previous study(1) evidence was presented that the infra-red absorption spectrum of the antistiffness factor obtained from sugar cane resembled the spectrum of stigmasterol more closely than any other spectrum of a

* Supported by the Armour Fund for Research in Muscular Disease and by a grant from the Muscular Dystrophy Association, Inc.

1. Rosenkrantz, H., Milhorat, A., Farber, M., and Milman, A., *Fed. Proc.*, 1951, in press.

large series of steroid compounds investigated. Purification and characterization of the natural antistiffness factor in the earlier work was accomplished by solvent partition and spectrophotometric analysis. The present paper is a continuation of this work.

Counter-current distribution was performed in a 24-plate Craig apparatus(2), a methanol-isooctane two-phase system being utilized(3). Solvent partition was applied to a purified concentrate† from sugar cane, the migration of the material being followed by dry weight determinations and ultra-violet absorption characteristics. On the basis of both the latter analytical technics, the concentrate was divided into 3 fractions, A, B, and C. Infra-red analysis of each fraction ascertained the relative purity and aided in characterization of each fraction.

The infra-red absorption data indicated that fractions A and C were free of each other and that fraction A probably was a sterol. The sterol nature of fraction A was further demonstrated by a positive Liebermann-Burchard reaction. Since fraction A gave also a mild, positive Rosenheim color reaction, the presence of some diene impurity was suggested and this was supported by the ultra-violet absorption spectrum of fraction A, which at high concentrations was nearly identical to those of ergosterol, 7-dehydrocholesterol and other $\Delta^{5,7}$-diene steroids(4). It was not concluded by Ross, van Wagtendonk and Wulzen(5), despite low extinction coefficients which they obtained, that the similar absorption spectrum of their antistiffness factor was due to a diene impurity.

The infra-red absorption spectrum of fraction A was utilized in screening a large series of steroid compounds, efforts being concentrated on stigmasterol and ergosterol derivatives of the dihydroergosterol type because of spectral similarities. The presence of a hydroxyl group was established by the 2.95 and 9.45 μ absorption bands (elemental analysis of the antistiffness factor by Ross, *et al.*(5) had demonstrated the presence of only one oxygen) and this hydroxyl group was assigned a β-configuration on the basis of digitonin precipitation. Infra-red absorption bands of moderate intensity near 6 μ in the spectrum of fraction A suggested either contamination by oxidation products, a conjugated double bond system (not in a benzene ring system) or a combination of both. The absorption bands between 9 and 11 μ strongly suggested that the infra-red spectrum of fraction A was closer to that of stigmasterol than to that of ergosterol derivatives, cholesterol derivatives, α-spinasterol or the steroidal hormones.

Sublimation of fraction B, a mixture of fractions A and C which contained most of the distributed material, resulted in further purification. The infra-red absorption spectrum of this sublimate was nearly identical to that of stigmasterol but the material contained some fraction C. A combination of absorption bands near 9.5, 9.8, 10.2, 10.3 and 10.4 μ were characteristic of stigmasterol and the sublimate but not of the other steroids studied. Absorption bands near 5.8, 13.7 and 13.9 μ indicated the presence of fraction C, the non-steroid component. A complete identification of the antistiffness factor from sugar cane as being stigmasterol was afforded upon receipt of a purified sterol isolated from sugar cane by the Armour Laboratories.† The infra-red absorption spectrum of this antistiffness sterol was identical with that of stigmasterol. Structural interpretations and the use of infra-red data in characterizing stigmasterol (the antistiffness factor) will be discussed in a subsequent paper.

After this work had been completed, we were informed that Drs. Emil Kaiser and

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3. Ulick, S., and Milhorat, A. T., *Science*, 1949, v110, 531.

† We wish to express our gratitude to the Eli Lilly Company for donation of the purified concentrates from sugar cane and to the Armour Laboratories for a sample of their purified sterol.

4. Morton, R. A., The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes, Adam Hilger, Ltd., London, 1942, Chap. II.

5. Ross, L. E., van Wagtendonk, W. J., and Wulzen, R., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 281.

6. Kaiser, E., and Wulzen, R., *Arch. Biochem.*, 1951, in press.

Rosalind Wulzen(6) on the basis of chemical and biological evidence other than infra-red spectroscopy, also concluded that the anti-stiffness factor obtained from sugar cane wax is stigmasterol.

Received January 23, 1951. P.S.E.B.M., 1951, v76.

Effect of Fecal Bacterial Activity on Rectal Temperature of Man. (18507)

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Claude Bernard(1) called attention to the fact that the rectal temperature was one of the highest to be found in the body. This observation has been confirmed by other investigators(2). It seemed possible that this elevation in rectal temperature might be due, in part at least, to bacterial activity in the rectum and rectosigmoid. Bazett(3) suggested such a possibility. If this hypothesis were true, reduction of the intestinal bacteria, as by an orally administered, poorly absorbed sulfonamide, might be expected to result in a decrease in rectal temperature. Ravdin *et al.*(5) and others have shown that the technic of succinylsulfathiazole administration such as was employed here will result in a marked reduction in the number of organisms present in the feces.

Methods. Five normal young adult males served as subjects. Each subject received, in 4 divided daily doses, succinylsulfathiazole orally, 25 mg per kilo of body weight per 24 hours. The drug was given in this manner for 7 days. The subjects' diets and physical activity remained approximately constant for the periods before, during, and after the drug administration. With the subjects supine, rectal temperatures were recorded for 30 minutes preceding the noon meal and in a constant relationship to the subjects' physical activity and bowel movements. Rectal temperature determinations were made by copper-constantan thermocouples inserted 8 cm into the rectum. Measurements were made 8 hours before the drug administration was begun and were continued throughout the course of the drug. Temperatures were recorded at 2-minute intervals by a modified Brown automatic recording potentiometer whose accuracy was within 0.05°C. The temperatures of the final 6 minutes of the daily observation periods were averaged to obtain the temperature of the individual for any one day.

In some instances, smears from fecal specimens were obtained at the time of the temperature measurements. From these smears gross estimates of the number of fecal bacteria present were made in the manner described by Dearing and Heilman(4).

Results. Although the fecal bacterial content decreased markedly following the succinylsulfathiazole ingestion, no significant changes in rectal temperatures were seen (Tables I and II).

Discussion. It is apparent that exothermic reactions associated with the fermentation processes of microorganisms in the lower colon and rectum are of minor importance in modifying the level of the rectal temperature in man. Since the metabolism of this portion of the gastro-intestinal tract is considered to be quite low the obvious factor involved in maintaining the temperature of the organ must be the temperature of the perfusing blood. The chief arterial supply of the lower 12 cm of the large intestine is derived from mesenteric branches of the aorta, all of which are within the abdominal cavity. The venous

1. Bernard, C., Lecons sur la chaleur animale, Paris, 1876.

2. Horvath, S. M., Rubin, A., and Foltz, E., *Am. J. Physiol.*, 1950, v161, 316.

3. Bazett, H. C., personal communication.

4. Dearing, W. H., and Heilman, F. R., *Proc. Staff Meeting of the Mayo Clinic*, 1950, v25, 87.

TABLE I. Effect of Succinylsulfathiazole on Level of Rectal Temperature in °C, as Recorded Daily in Adult Men.*

Subject	Day 0	1	2	3	4	7
E.	37.71	37.50	37.62	37.63	37.56	37.52
F.	37.66	37.60	37.53	37.54	37.38	37.60
H.	37.40	37.40	37.53	37.41	37.33	37.59
B.	37.56	37.45	37.36	37.55	37.72	37.31
S.	37.20	37.48	37.28	37.39	—	37.26
Avg	37.51	37.49	37.48	37.50	37.50	37.47

* Day 0 is 8 hr before beginning of oral administration of succinylsulfathiazole 25 mg per kg body wt per 24 hr. This level of drug dosage was continued throughout days 1 to 7, inclusive.

TABLE II. Organisms Present in Feces.*

Subject	Days of drug admin.	Smears g +	g —	Yeasts
F.	0	4+	4+	1+
	7	2+	2+	1+
B.	0	4+	4+	2+
	7	3+	2+	1+
E.	0	4+	4+	0
	7	1+	1+	0

* Films were made directly from the feces and stained by Gram's method. Their numbers were estimated on a basis of 1+ to 4+(4). Days of drug administration refers to number of days subjects had been taking succinylsulfathiazole orally before the fecal smears were made.

drainage of the rectum and anal canal is effected by the superior, middle, and inferior hemorrhoidal veins. The superior belongs to the portal system; the middle and inferior being a part of the caval network. As in the case of the arteries, the rectal veins are also within the body cavity. While there appears to be a gradient of temperature within the rectum, this is usually of the order of magnitude of 0.1 to 0.2°C in the lower 6 inches of the alimentary canal(6). A very localized site on the posterior wall of the colon some 8 inches above the anal orifice may be as much as 0.5°C cooler than the rest of the organ. The cooling of this small portion of the intestine could be attributed to the presence nearby of veins draining certain areas of the body surface.

Eichna(7) suggested that the femoral arterial temperature provided a better index of deep body temperatures (left ventricle blood temperature) than the rectal temperature. However, Eichna(7) consistently found a lower temperature in the femoral artery as compared with that measured in the rectum. This would indicate that the blood in the femoral had been cooled somewhat during its exit from the abdominal cavity and that it therefore did not represent deep body temperature. That this cooling of the arterial blood in its departure from the more central parts of the body is real is suggested by the investigation of thermal gradients in dogs by Horvath *et al.*(2). These workers found a consistent fall in arterial temperature as the blood passed from the iliacs into femoral. It is possible that the rectal temperature may lag behind simultaneously observed blood temperature but it is doubtful that this lag is of much significance, particularly in resting states. Even in very dynamic states such as in individuals riding vigorously on a bicycle ergometer, the rectal temperature did not differ appreciably from simultaneously recorded central vascular temperatures(8).

Summary. 1. Succinylsulfathiazole was administered orally to 5 normal adults and resulted in a marked decrease in the number of bacteria present in their feces. 2. There were no significant changes in the rectal temperatures of the subjects concomitant with the fall in their fecal bacterial content.

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Received January 29, 1951. P.S.E.B.M., 1951, v76.